PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:	Examiner: B. Whiteman
BARNETT et al.	Group Art Unit: 1635
For: **POLYNUCLEOTIDES ENCODING ANTIGENIC HIV TYPE C POLYPEPTIDE, POLYPEPTIDES AND USES THEREOF**	Confirmation No.: 6738
Serial No.: 09/475,704	**DECLARATION PURSUANT TO 37 C.F.R. § 1.132**
Filed: December 30, 1999	
Atty. Docket No.: PP01631.002 (2302-1631)	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313

Sir:

I, Jeffrey Ulmer, hereby declare as follows:

1. I received my Bachelors of Science Degree in Chemistry from the University of Regina in 1978 and a Doctorate of Philosophy Degree in Biochemistry from McGill University in 1985.

2. I am currently Senior Director at Chiron Corporation and have been at Chiron since 1998. Before joining Chiron, I was Senior Research Fellow at Merck. Additional details regarding my background and qualifications can be found in the accompanying copy of my *Curriculum Vitae* (Exhibit A).

3. I have reviewed pending Patent Application Serial No. 09/475,704, for "POLYNUCLEOTIDES ENCODING ANTIGENIC HIV TYPE C POLYPEPTIDE, POLYPEPTIDES AND USES THEREOF" by Barnett, et al., (hereinafter "the specification")

and the currently pending claims. I have also reviewed the Office Action dated November 17, 2003. Therefore, I am familiar with the issues raised by the Examiner in the Office Action.

4. I understand that the pending claims are directed to expression cassettes comprising nucleotide sequences that encode HIV *Gag* polypeptides, where the polypeptides elicit a *Gag*-specific immune response. Further, the *Gag*-encoding nucleotide sequence must exhibit at least 90% identity to the sequences of SEQ ID NOs:1-4. It is further my understanding that the claims are also directed to cells comprising these polynucleotides and to methods of generating a *Gag*-specific immune response in a subject using the claimed polynucleotide sequences.

5. It is my opinion that, as a technical matter, a skilled worker could have readily made and used the compositions and methods of the pending claims in light of the specification, together with the common general knowledge, tools and methods available in December 1999. I base this opinion on the data and facts set forth below; however, I call attention to the fact that it was considered routine experimentation at the time of filing to determine a sequence having (i) at least 90% sequence identity to SEQ ID NO:1-4 and (ii) encoding an immunogenic Gag polypeptide; to express such polynucleotides in stem cells or their progenitors; to deliver in a variety of ways such polynucleotides to generate an immune response in a subject. In addition, in drawing my conclusions, I have considered the nature of the claims, the quantity of experimentation required to practice the subject matter of the claims, the existence of working examples, the direction present in the specification, the state of the field at the time the application was filed and the level of skill in the art.

6. At the outset, I note that the term "skilled worker" with a routine level of skill in the field of molecular biology, immunology and nucleic acid delivery in December 1999 had a Ph.D. degree and two or more years of post-doctoral training. In view of my training and experience, I am currently, and was in December of 1999, such a skilled worker.

7. In December 1999, the quantity of experimentation required to identify sequences exhibiting 90% identity to any given sequence, for example SEQ ID NOs:1-4, was quite low. For example, BLAST software programs were commonly known and readily available on the Internet at this time. This set of programs allows for an easy alignment and determination of

percent identity as between any sequences. The skilled worker could have easily used the BLAST or any number of other similar programs to determine the percent identity between sequences (in this case between any given sequence and those presented SEQ ID NOs:1-4). The specification also provides extensive guidance in this regard, for example, pages 17-19 and Examples of the specification. Working examples are also provided -- indeed, the specification provides additional sequences falling within the scope of the claims 1 and 2 (SEQ ID NO:20) and claims 3 and 4 (SEQ ID NO:21). Furthermore, the skilled worker could have readily generated any sequence falling within the scope of the claims using routine methods, for example by utilizing PCR to generate sequences, by introducing point mutations and the like. Thus, it is my opinion that it would have required only routine experimentation to determine sequences falling within the 90% identity, as claimed.

8. In addition, the specification provides significant direction for evaluating whether sequences having 90% identity to SEQ ID NO:1-4 encode an immunogenic Gag polypeptide. Those of us working in the field of gene delivery and immunology are well versed in the various tests for determining immunogenicity, which include computer analysis of sequences, comparison to known immunogenic sequences as well as functional tests (*e.g.*, ELISAs, CTL assays and others described in the Examples of the specification). Gag antigens or antibodies recognizing Gag antigens had long been used to test for Gag-stimulated immune responses (*e.g.*, immunoassays). ELISPOT and flow cytometry assays for testing cellular immunity were also well known at the time of filing.

9. Furthermore, the state of the art in December 1999 was quite sophisticated with regard to determining both sequence identity and evaluating immunogenicity. I have described above some of the tools, programs and methods available in the field of recombinant nucleic acid technology in December 1999 and many other examples of homologous nucleic acid molecules that encode immunogenic proteins were also available. Therefore, it is my opinion that, following the guidance of the specification, a scientist could have readily made and used polynucleotide sequences that exhibit at least 90% sequence identity to SEQ ID NO:1-4 and which encode an immunogenic HIV Gag polypeptide.

10. Preparing polynucleotides encoding immunogenic Gag polypeptides in December 1999 was a predictable art. There is no doubt that a skilled worker would have been able to make and use sequences exhibiting 90% identity to SEQ ID NO:1-4 and encoding an immunogenic polypeptide. Even if a rare construct were inoperable for some reason (*e.g.*, it wasn't immunogenic), the skilled worker would have readily modified the construct according to the alternatives available at the time and described in the specification. In other words, to the skilled worker, an inoperable construct would itself be a useful starting material for other operable constructs. Essentially all molecules that fall within the claims would be useful for making or using defining technical features of the claims, *i.e.*, nucleotide sequences having 90% sequence identity to SEQ ID NO:1-4 and which encoded an immunogenic HIV Gag polypeptide.

11. Similarly, the specification as filed clearly provides ample guidance on how to make and use *Gag* polypeptides to generate a *Gag*-specific immune response (humoral and/or cellular) in a subject by administering the claimed sequences (or polynucleotides encoded by those sequences). (See, Examples 4 and 7). Indeed, in December 1999, it was predictable and routine to evaluate whether an immune response was generated against a polypeptide antigen encoded by an administered polynucleotide, for example using the techniques and tools described above in paragraph 8. Furthermore, the skilled worker would know that generating an immune response does not necessarily mean that the subject will be immunized – *i.e.*, protected against HIV infection or derive some therapeutic benefit. The skilled worker would also have known that immune responses are useful for numerous scientific purposes, such as laboratory assays, preparing reagents for virologic and immunologic studies, quantifying and analyzing immune responses, and preparation of diagnostic kits. Therefore, a skilled worker would have known that the claimed sequences could be used for additional scientific purposes other than seeking protective immunity or a therapeutic benefit. In view of the guidance in the specification, the predictability and state of the art, and high level of the skilled worker, it is plain that it would have been routine to administer a polynucleotide and evaluate whether or not an immune response to the encoded polypeptide was generated in the subject.

12. Experiments conducted after filing of the application also demonstrate that expression cassettes that include modified HIV *Gag*-encoding sequences induce potent *Gag*-specific immune responses. These experiments are summarized in zur Megede et al. (2003) *J. Virol.* 77(11):6197-6207, attached hereto as Exhibit B. As shown in Exhibit B, we generated modified *Gag*-encoding sequences from subtype B isolates of HIV using the protocols described in the specification. (*See*, Example 1). Also using techniques set forth in the specification, we inserted these modified *Gag*-encoding sequences into an expression cassette such that they are operably linked to a promoter. These expression cassettes were administered to living animals and immunogenicity evaluated, using the protocols set forth in the specification. Our results establish that "all of the sequence-modified pol and gagpol plasmids expressed high levels of *Gag*-specific antigens in a Rev-independent fashion and we were able to induce potent *Gag*-specific T- and B-cell responses..." (Abstract of Exhibit B). In light of these results, I conclude that modified HIV *Gag*-encoding sequences can be inserted into expression cassettes such that they are operably linked to a promoter and that these expression cassettes elicit *Gag*-specific immune responses. I also conclude that a variety of sequences exhibiting 90% homology to each other are similarly effective. Furthermore, because *Gag*-encoding sequences can be obtained from any HIV isolate and modified as described in the specification, the results we presented in Exhibit B with regard to subtype B sequences are equally applicable to modified polynucleotides obtained from subtype C isolates, as claimed.

13. It would have also been routine to express the claimed *Gag*-encoding polynucleotides in stem cells or lymphoid progenitor cells. The guidance in the specification in this regard is extensive. In addition, the level of skill in this field was very high at the time of filing, the state of the art sophisticated and the experimentation needed to get expression in lymphokine cells (such as stem cells and lymphoid progenitor cells) was routine using standard vectors (*e.g.*, widely available plasmids that include promoters and other control elements). Even a reference cited in the Office Action makes it clear that heterologous HIV polypeptide-encoding sequences can readily be introduced into and expressed in stem cells:

> Other areas where gene transfer into hematopoietic cells is being investigated include human immunodeficiency virus (HIV) infection ... the importance of

> these studies cannot be over emphasized as they provide 'proof-in-principle' that gene-marked cells can survive and be expressed for extended periods of time once re-introduced into the host. (Prince, *Pathology* 30:335-347 at page 340, left column, emphasis added).

Therefore, the specification teaches a skilled worker how to express the claimed *Gag*-encoding sequences in stem cells or progenitors of lymphoid cells.

14. Furthermore, I believe that, following the teachings of the specification and guidance of the art, a skilled worker could have readily administered the claimed nucleic acids by a variety of modes including intramuscular, intradermal, mucosal and the like. The quantity of experimentation required to use alternatives to intramuscular delivery routes was quite low in December 1999. A skilled worker could have easily administered polynucleotides by a variety of routine methods known at the time of filing. For example, administration of polynucleotides encoding HIV antigens via intradermal and mucosal modes is described in Shiver et al. 1997 *Vaccine* 15:884-887 (Exhibit C) and Durrani et al. 1998 *J. Immunol. Methods* 220:93-103 (Exhibit D). These references are clearly representative of the high level of skill in the art and the fact that non-intramuscular modes of administration were considered predictable in December 1999 -- many of the examples gene delivery modes were also known. Furthermore, at the time of filing, it was known in the art that administration of polynucleotide vaccines by diverse routes such as intradermal, transdermal, intranasal, oral and the like did not require special modifications to the coding sequence of the polynucleotide plasmid construct itself. The specification provides significant direction in these regards as well, for example on page 61 of the specification. Therefore, a skilled worker would have found the claimed expression cassette and sequences at least 90% identical to it to be useful for generating an immune response using diverse routes and methods. Thus, to the skilled worker, administering the claimed polynucleotides by any number of delivery routes would have been routine and required only minor experimentation.

15. It is also my opinion that the specification as filed clearly conveyed to a typical scientist that the inventors had in their possession the invention set forth in the claims (see paragraph 4 above). By "in their possession," I mean that the inventors contemplated the

polynucleotides, cells and methods as set forth in the claims and that they had, using the specification and information available to a typical scientist, a practical way of making such molecules and practicing such methods. Thus, I believe that a typical scientist would have understood the specification clearly described all of the various aspects of the claims. I base this belief on the facts set forth herein.

16. First, the specification unambiguously and clearly describes at the time of filing, the structure of the claimed biomolecules. The claims indicate that the polynucleotide must exhibit 90% homology to a reference sequence and it was widely known how to determine sequence identity to any length polynucleotides. Such methods are described in detail in the specification, for example, on pages 19-21 of the specification. (see, also, paragraph 7 above). Therefore, it is my opinion that the specification describes any sequence exhibiting 90% sequence identity to SEQ ID NOs:1-4.

17. Second, at the time the specification was filed, it would have been clear to a typical scientist that the inventors' specification fully described and contemplated the function of the claimed biomolecules, namely that the claimed polynucleotides encoded Gag polypeptides that elicit a *Gag*-specific immune response. Methods of testing Gag polypeptides for their ability to elicit *Gag*-specific immune responses were well known at the time of filing and are demonstrated, for example, in Exhibit B. In sum, based on the disclosure of the specification and the level of knowledge of a typical scientist regarding sequence identity, and testing for immunogenicity, I believe that the specification as filed clearly conveys that the applicants had invented the expression cassettes as set forth in the claims.

18. Third, the specification unambiguously and clearly describes at the time of filing, the correlation between structure of the claimed biomolecules and their immunogenic function. The amino acid sequences of many *Gag* antigens were known at the time of filing and, in addition, methods of determining others were clearly available as of the date the application was filed. *See, e.g.*, page 12, lines 19-27 of the specification; Exhibit E (Parker et al. (1994) *J. Immunol.* 152:163-175; Exhibit F (Johnson et al. (1991) *J. Immunol.* 147:1512-1521). In particular, Exhibit E evidences that it was routine to those of working in the field to predict T

cell epitopes from amino acid sequence and Exhibit F demonstrates identification of *Gag* T cell epitopes prior to December of 1999. Furthermore, those of us working in this field knew, at the time of filing, that any given antigen can tolerate a number of amino acid substitutions while still retaining its immunogenic function. In other words, a particular amino acid sequence is not required in order to elicit a *Gag*-specific immune response. Rather, one would expect that a multitude of *Gag* polypeptides, having different amino acid sequences, would function to generate specific an immune response in a subject. Thus, it would have been clear to the skilled worker that the specification describes the correlation between the structure and function set forth in the claims.

19. In view of the foregoing facts regarding the routine nature of experimentation required to make and use the claimed constructs, the extensive direction provided by the specification, the straightforward nature of the invention, the presence of working examples, the high level of the skilled worker, the sophistication of the art, and the predictability (*e.g.*, of determining sequences identity and immunogenicity) of the art, it is my unequivocal opinion that the specification enabled, in December 1999, a skilled worker to make and use the subject matter of the claims. Similarly, in view of the detailed description in the specification and state of the field at the time of filing, it is my opinion that the specification more than adequately conveys that the inventors had possession of the claimed polynucleotides, expression cassettes, vectors, cells and methods of generating immune responses at the time of filing in December 1999.

20. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

1/19/04
Date

Jeffrey Ulmer, Ph.D.

CURRICULUM VITAE

1/6/04

PERSONAL

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EDUCATION

School	Date	Major/Minor	Degree
McGill University	1985	Biochemistry	Ph.D.
Luther College, Univ. of Regina	1978	Chemistry/Biochemistry	B.Sc. (Honours)

ACADEMIC AND PROFESSIONAL HONORS

1983	Hugh Edmund Burke Medical Research Award
1981	Multiple Sclerosis Society of Canada Postgraduate Scholarship
1980	Natural Sciences and Engineering Research Council of Canada Postgraduate Scholarship
1978	Graduated with Distinction
1978	Society of Chemical Industry Merit Award

PROFESSIONAL RESEARCH EXPERIENCE

2003	Head, Immunology and Cell Biology, Chiron Corporation.
2000	Senior Director, Vaccines Research, Chiron Corporation.
1998	Director, Vaccines Research, Chiron Corporation.
1996	Senior Research Fellow, Department of Virus and Cell Biology, Merck Research Laboratories.
1993	Research Fellow, Department of Virus and Cell Biology, Merck Research Laboratories.
1990	Senior Research Biochemist, Department of Cancer Research, Merck Research Laboratories.
1988	Associate Research Scientist, Department of Cell Biology, Yale University School of Medicine.

PUBLICATIONS

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2. Ulmer, J.B. and Braun, P.E. 1984. "*In vivo* phosphorylation of myelin basic proteins in developing mouse brain: Evidence that phosphorylation is an early event in myelin formation". **Dev. Neurosci.** 6, 345-355.
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Expression and Immunogenicity of Sequence-Modified Human Immunodeficiency Virus Type 1 Subtype B *pol* and *gagpol* DNA Vaccines

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Control of the worldwide AIDS pandemic may require not only preventive but also therapeutic immunization strategies. To meet this challenge, the next generation of human immunodeficiency virus type 1 (HIV-1) vaccines must stimulate broad and durable cellular immune responses to multiple HIV antigens. Results of both natural history studies and virus challenge studies with macaques indicate that responses to both Gag and Pol antigens are important for the control of viremia. Previously, we reported increased Rev-independent expression and improved immunogenicity of DNA vaccines encoding sequence-modified Gag derived from the HIV-1_{SF2} strain (J. zur Megede, M. C. Chen, B. Doe, M. Schaefer, C. E. Greer, M. Selby, G. R. Otten, and S. W. Barnett, J. Virol. 74: 2628-2635, 2000). Here we describe results of expression and immunogenicity studies conducted with novel sequence-modified HIV-1_{SF2} GagPol and Pol vaccine antigens. These Pol antigens contain deletions in the integrase coding region and were mutated in the reverse transcriptase (RT) coding region to remove potentially deleterious enzymatic activities. The resulting Pol sequences were used alone or in combination with sequence-modified Gag. In the latter, the natural translational frameshift between the Gag and Pol coding sequences was either retained or removed. Smaller, in-frame fusion gene cassettes expressing Gag plus RT or protease plus RT also were evaluated. Expression of Gag and Pol from GagPol fusion gene cassettes appeared to be reduced when the HIV protease was active. Therefore, additional constructs were evaluated in which mutations were introduced to attenuate or inactivate the protease activity. Nevertheless, when these constructs were delivered to mice as DNA vaccines, similar levels of $CD8^+$ T-cell responses to Gag and Pol epitopes were observed regardless of the level of protease activity. Overall, the cellular immune responses against Gag induced in mice immunized with multigenic *gagpol* plasmids were similar to those observed in mice immunized with the plasmid encoding Gag alone. Furthermore, all of the sequence-modified *pol* and *gagpol* plasmids expressed high levels of Pol-specific antigens in a Rev-independent fashion and were able to induce potent Pol-specific T- and B-cell responses in mice. These results support the inclusion of a *gagpol* in-frame fusion gene in future HIV vaccine approaches.

The AIDS pandemic caused by human immunodeficiency virus type 1 (HIV-1) is believed to have cost 3.1 million lives in the year 2002 alone, with over 42 million people believed to be infected worldwide (http://www.unaids.org/worldaidsday/2002/press/Epiupdate.html). At present, 20 years after the discovery of HIV/AIDS, no effective HIV vaccine has been identified and few candidates have advanced beyond early-phase clinical trials (20). While effective drug therapy is available in developed parts of the world, it is financially out of reach for most of the world's population of infected individuals. It is thus widely believed that an efficacious prophylactic vaccine is critical for the control of the global spread of HIV/AIDS. Furthermore, therapeutic vaccine approaches in combination with drug therapy, which allow patients to be off drugs for extended periods of time, also hold great promise for those already infected (18, 45).

While the primary focus for first-generation HIV vaccines was the induction of neutralizing antibodies using HIV envelope (Env)-based approaches, more recently, the focus has extended to the induction of $CD8^+$ cytotoxic T-lymphocyte (CTL) responses against conserved internal viral antigens such as Gag and Pol (17). This shift was a result of studies of natural infections, long-term nonprogressors, and exposed uninfected individuals that have, in multiple studies, demonstrated an inverse correlation between the potency and breadth of $CD4^+$ and $CD8^+$ T-cell responses and HIV disease progression (7, 8, 14, 29, 31, 33, 44, 46). Moreover, vaccine approaches specifically designed to induce strong cellular immunity recently have shown promising results in nonhuman primate vaccine challenge models (2, 5, 49). In these studies, the induction of strong $CD8^+$ T-cell responses against Gag in vaccinated macaques appeared to result in decreased viremia, morbidity, and mortality when animals were subsequently challenged with pathogenic simian/human immunodeficiency viruses. Nevertheless, this strategy of using gene-based vaccines alone to induce $CD8^+$ T-cell responses does not appear to protect monkeys from infection and the challenge virus was able to eventually escape immune control, resulting in increased viremia and its sequelae (4, 24).

Interestingly, the use of prime-boost immunization strategies, including those that use Env antigens as the protein in

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several of these CTL-based vaccines, has repeatedly been shown to improve the degree of protection observed (23, 36, 37, 42, 43). Whether this is due to the priming of protective B- or T-cell responses has not been elucidated in these studies. In addition to the use of prime-boost strategies, the use of multiple genes in the vaccine to increase the number of potential T-cell epitopes has also improved the outcome after a virus challenge over that achieved with a single- or double-gene vaccine (2, 30). Therefore, the overall goal of our program has been to achieve the greatest breadth of cellular immunity directed to multiple HIV antigens in combination with broad neutralizing antibody responses, an approach that may be more successful at blocking infection than has been previously observed.

The goal of the present study was to evaluate the expression and immunogenicity of novel vaccine antigens based on portions of the HIV-1 Pol polyprotein administered alone or in combination with Gag. Pol is a conserved protein of HIV-1, and cross-clade CTL responses against Pol epitopes have been detected in both HIV-infected and exposed but uninfected individuals (6, 7, 47). The inclusion of the *pol* gene in the form of the *gagpol* precursor in earlier vaccine trials with humans and nonhuman primates was most likely suboptimal with regard to inefficient expression of the Pol antigen. The expression levels of the Pol protein generally are low during natural infection because of the frameshift required for translation of *pol* coding sequences. This mode of Pol expression results in an up to 95% reduction in Pol protein compared to Gag (27, 53). To increase Pol expression, the frameshift between *gag* and *pol* can be removed, resulting in equimolar or nearly equimolar expression of Gag and Pol whereas the secretion of virus-like particles (VLP) is impaired (28, 40). To evaluate the potential antigenic competition between Gag and Pol if they are encoded in one expression cassette, various expression cassettes were designed and tested with the antigens encoded on single or multigenic expression plasmids. Another consideration was the possible cytotoxic effect of the active viral protease and possible effects of the active protease on Gag and Pol antigen expression levels. Therefore, mutations known to attenuate or inactivate HIV-1 protease (32) were introduced. Additional safety features introduced into the *pol* expression cassette included the removal of integrase and the mutation of the reverse transcriptase (RT) to remove these potentially deleterious enzymatic activities.

Plasmid DNA vaccines encoding these sequence-modified *gag*, *pol*, and *gagpol* genes were evaluated for expression in vitro after transient transfection of 293 cells and subsequently in dose titration immunogenicity studies performed with mice. Overall, the cellular immune responses against Gag induced by the various multigenic Gag- and Pol-expressing plasmids were similar to those induced by the plasmid encoding Gag alone. All of the sequence-modified *pol* and *gagpol* plasmids expressed high levels of Pol-specific antigens in a Rev-independent fashion and were able to induce potent Pol-specific T-cell responses in mice. Moreover, removal of the frameshift between *gag* and *pol* resulted in increased expression of Pol and increased RT-specific immune responses, as expected. Lastly, while the activity of protease appeared to have an inhibitory effect on the expression of Gag and Pol antigens in vitro, the immunogenicities of constructs encoding active protease did not appear to be reduced in mice. The $CD8^+$ T-cell responses against Gag- and RT-specific epitopes, as measured by flow cytometric analysis of gamma interferon (IFN-γ)-producing cells, were comparable for all constructs regardless of the level of protease activity. These results support the inclusion of a sequence-modified in-frame *gagpol* fusion cassette in future HIV vaccine approaches.

MATERIALS AND METHODS

Plasmid DNA cassettes. A panel of expression cassettes based on the amino acid sequences of HIV-1_{SF2} subtype B Gag and Pol antigens was designed with sequence modifications as described previously (22, 56). All gene cassettes were cloned into eukaryotic expression vector pCMVKm2, which contained the cytomegalovirus immediate-early enhancer-promoter and the bGH terminator (Chiron Corporation, Emeryville, Calif.) (12). To further enhance the translation efficiency of all expression cassettes, an optimal "Kozak" consensus sequence (GCCACC) for initiation of translation was inserted (34). *gag*-only plasmid pCMVKm2.GagMod.SF2 (GenBank accession no. AF201927) and *gagprotease* cassettes GP1 and GP2 (pCMVKm2.GagProt.SF2; GenBank accession no. AF202464 and AF202465) have been described previously (56).

The entire integrase coding sequence in *pol* was deleted for safety reasons, and the catalytic triad and primer grip regions of the RT coding sequences were deleted to inactivate these enzymatic activities (39, 41). The construct gagFSpol was based on the GP2 cassette but was extended for *pol* up to the RNase H coding sequences. For the gagpol and gag-complete-pol (gagCpol) cassettes, the frameshift region was removed by insertion of an extra T nucleotide at the p1 "slippery sequence" (TTTTTTA) in order to express the *gag* and *pol* genes in frame. The *pol* region included $p1p6^{Pol}$ coding sequences up to RNase H for gagpol. To include $p1p6^{Gag}$ and for optimal processing of Gag and Pol by the protease, the *p2p7p1p6* fragment was added to get gagCpol (see Fig. 1). The constructs gagRT and gagprotInaRT expressed fusion proteins of $p55^{Gag}$ and either $p66^{RT}$ or $p10^{protease}$ plus $p66^{RT}$. Furthermore, gene cassettes for the expression of $p66^{RT}$ alone and proteaseRT and $p2p7^{Gag}$ plus $p1p6^{Pol}$ (p2pol) were also included. When indicated, the protease in some constructs was either attenuated (Att) or inactivated (Ina) by the introduction of specific point mutations (32) with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, Calif.).

Testing for in vitro expression. Human kidney 293 cells (no. 45504; American Type Tissue Collection, Manassas, Va.) were plated 1 day prior to transfection at a density of 5×10^5 cells per 35-mm-diameter well (Corning, Acton, Mass.) and transfected with endotoxin-free purified plasmid DNA (Qiagen, Valencia, Calif.). For the transfections, 2 μg of each plasmid DNA was mixed with Mirus TransIT-LT1 Polyamine transfection reagent (PanVera, Madison, Wis.). The cells were incubated with 2 ml of 10% Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, Calif.) per well for 48 and 72 h, and the supernatants and lysates were then harvested for further analysis. Quantitation of $p24^{Gag}$ protein in cell supernatants and lysates was performed with the Coulter p24 Antigen Capture enzyme-linked immunosorbent assay (ELISA; Coulter Corporation, Miami, Fla.). The Western blot assay for Gag and Pol expression analysis was done by using 4 to 12% Bis-Tris sodium dodecyl sulfate-polyacrylamide gels (Invitrogen) and then transfer onto 0.2-μm-pore-size nitrocellulose (Invitrogen). Prestained full-range rainbow marker (Amersham, Piscataway, N.J.) and recombinant HIV-1 $p24^{Gag}$, $p55^{Gag}$ (Chiron), and $p66^{RT}$ (Protein Sciences, Meriden, Conn.) proteins were used as the size standard and positive controls, respectively. For detection of Gag proteins by immunostaining, membranes were incubated with HIV-1-positive human serum at a dilution of 1:400. For Pol proteins, an anti-$p66^{RT}$ monoclonal antibody (MAb; 1:200; Fitzgerald, Concord, Mass.) and pooled mouse serum (1:400) against $p66^{RT}$ (Chiron) were used. Secondary antibodies (1:20,000) were anti-human or anti-mouse immunoglobulin G (IgG) conjugated to horseradish peroxidase (Pierce, Rockford, Ill.). Detection was performed by using the enhanced chemiluminescence substrate (Amersham). The predicted molecular weights of the various expression cassettes tested were calculated from the predicted amino acid sequences by using MacVector software (Oxford Molecular Ltd.).

Immunization of mice. To evaluate the relative potencies of the immune responses induced by the different constructs, female CB6F1 or C3H/HeN mice, 6 to 8 weeks old, were immunized bilaterally in the tibialis anterior muscles with 100-μl volumes of endotoxin-free plasmid DNA in isotonic saline (50 μl per site). The DNA concentrations of the test plasmids were adjusted to provide equal molar quantities of Gag or Pol at a given DNA dose. Furthermore, all DNA of

TABLE 1. Overview of mouse studies

Expt no.	Fig. no.	Mouse strain	Vaccines[a]	Immunization (day[s])	rVV challenge (day)	Blood collection (days)
1	4	CB6F1	a, b, c	0	28	0, 28, 33
2	5A, 6B	CB6F1	a, d, f, g, h	0	28	0, 28, 33
3	5B	CB6F1	a, d, f, g, h	0, 28	None	0, 28, 42
4	6A	CB6F1	a, b, c	0, 28	None	0, 28, 42
5	7A	C3H/HeN	e, f, g, i, k	0	28	0, 28, 33
6	7C	C3H/HeN	e, f, g, i, k	0, 28	None	0, 28, 42
7	7B	C3H/HeN	d, f, g, h, l	0	28	0, 28, 33
8	7D	C3H/HeN	d, f, g, h, l	0, 28	None	0, 28, 42

[a] Vaccines: a, gag; b, GP1; c, GP2; d, gagFSpol; e, gagRT; f, gagprotInaRT; g, gagCpolIna; h, gagCpol; i, RT; k, protInaRT; l, p2polIna.

<10 μg were adjusted to 10 μg by using noncoding vector pCMVKm2 as carrier DNA to avoid possible negative effects on immune potency that have been observed at low DNA doses (G. R. Otten, unpublished results). Table 1 contains a summary of the mouse studies performed and the immunization regimens used.

Measurements of antibody responses to $p24^{Gag}$. Plates (96 wells; Corning) were coated with 100 μl of recombinant HIV-1_{SF2} $p24^{Gag}$ antigen (Chiron) at a concentration of 2 μg/ml in 50 mM borate buffer, pH 9. Sera were diluted 1:25 and then serially diluted threefold in dilution buffer containing 1% casein as a blocking reagent. Pooled anti-$p24^{Gag}$ antibody-positive mouse sera served as both positive controls and assay standards. All sera were incubated for 1 h at 37°C, washed, and incubated with a 1:20,000 dilution of goat anti-mouse IgG plus IgM peroxidase conjugate (Pierce) for 1 h at 37°C. After washing of the plates, the tetramethylbenzidine substrate (Pierce) was added to each well and the reaction was stopped after 30 min by addition of 1 M H_3PO_4. The plates were read on an ELISA reader (312e; Bio-Tek Instruments, Inc., Winooski, Vt.) at 450 nm with a reference wavelength of 600 nm. The calculated titers are the reciprocal of the dilution of serum at a cutoff optical density of 0.4.

Challenge of immunized mice with recombinant vaccinia viruses (rVVs) expressing Gag or Pol. Challenge of *gag* DNA-primed mice with rVV expressing HIV-1_{SF2} GagPol (with frameshift) (B. Doe and C. Walker, Letter, AIDS **10:** 793-794, 1996) can enhance humoral and cellular immune responses to Gag compared to those observed after DNA immunization alone (Otten, unpublished). Thus, the rVVgagpol challenge model can provide a useful means by which to obtain quantitative measurements of antigen-specific $CD8^+$ T-cell function (Otten, unpublished). Mice were challenged 28 days postimmunization with an intraperitoneal injection of 10^7 PFU of rVV. Spleens were removed 5 days later, and spleen cells were isolated for further evaluation in an intracellular cytokine-staining (ICS) assay (described below). An rVV expressing HIV-1_{SF2} Pol was constructed to allow application of this challenge model for the measurement of Pol-specific T-cell responses. Because of the frameshift in *gagpol*, the expression of Pol was insufficient if rVVgagpol was used. The complete codon-optimized *pol* sequence, with the exception of *integrase*, was used. Protease and RT were left functional. The gene was cloned into the shuttle vector pSC11 (11) via *Xma*C1 and *Hin*dIII sites, and rVV expressing Pol was generated as described for rVVgagpol.

ICS for Gag- and Pol-specific IFN-γ-producing $CD8^+$ lymphocytes. Stimulation and staining of isolated spleen cells were done as described previously (56). Briefly, spleens were harvested 2 weeks post second DNA immunization or 5 days post rVV challenge and single-cell suspensions were prepared. Nucleated spleen cells (10^6) were cultured in duplicate at 37°C in the presence or absence of 10 μg of p7g peptide per ml (Doe and Walker, letter) for Gag or by using the RT39-47_{SF2} peptide TEMEKGEKI (35) for the stimulation of Pol-specific $CD8^+$ cells. Unstimulated cells plus spleen cells from naive mice were used as background and negative controls. The background values were generally very low, between 0.01 and 0.1% of IFN-γ-secreting $CD8^+$ cells. After 5 h, cells were washed, incubated with anti-CD16/32 (Pharmingen, San Diego, Calif.) to block Fcγ receptors, fixed in 1% (wt/vol) paraformaldehyde, and stored overnight at 4°C. On the following day, cells were stained with fluorescein isothiocyanate-conjugated CD8 MAb (Pharmingen), washed, treated with 0.5% (wt/vol) saponin (Sigma), and then incubated with phycoerythrin-conjugated mouse IFN-γ MAb (Pharmingen) in the presence of 0.1% (wt/vol) saponin. Cells were then washed and analyzed on a FACScalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif.).

Statistical analysis of Gag- and Pol-specific IFN-γ-producing $CD8^+$ T-cell responses. For analysis of the relative $CD8^+$ T-cell responses in the mouse immunogenicity studies, a regression analysis was performed. Each regression analysis began with a single regression model incorporating indicator variables to allow for individual intercepts and slopes specifically for each treatment. The model is $Y_i = \beta_0 + \beta_{0i}\delta_i + \beta_1 x + \beta_{1i}\delta_i x + \epsilon$, where $i = 1 \ldots$no. of treatments. Here Y_i is the $\log_{10}$ background-corrected percentage of cells showing a positive CD8 IFN-γ response for peptide treatment group i and HIV DNA vaccine dose level x. The intercept for each treatment is the overall intercept, β_0, plus an additional term, β_{0i}, for treatment i. The slope for each treatment is $\beta_1 x$ plus an additional term, $\beta_{1i}\delta_i x$. The δ_i values are indicator variables that equal 1 for treatment i and are 0 otherwise. The model was iteratively reduced by removing first nonsignificant slope terms, those with $P > 0.05$, and then nonsignificant intercept terms, those with $P > 0.05$, in the reduced-slope model. The result was a final regression model with only the significant slope and intercept terms, those with $P < 0.05$. This model-building process was repeated for each of seven experiments, corresponding to Fig. 4, 5A and B, and 7A to D. Scatter plots for each figure including the significant regression model equations for each treatment were plotted by using SPlus 2000.

RESULTS

Construction of novel *gag*- and *pol*-derived expression cassettes. Previously, we reported on the construction and characterization of a sequence-modified Gag plasmid that was found in several studies to be a potent inducer of Gag-specific immune responses (38, 56). In the present work, we sought to broaden the spectrum of viral epitopes represented in our DNA vaccine approach (without introducing a reduction of Gag-specific immune responses) through the addition of Pol coding sequences. For this purpose, we designed and evaluated several novel *gag* and *pol* expression cassettes. A summary of the sequence-modified gene cassettes evaluated here is shown in Fig. 1. The constructs GagMod (gag), GP1, and GP2 were described and characterized previously but are included for comparison (56). The gene cassette gagFSpol was based on GP2 with an extension of Pol including the $p66^{RT}$ coding region but without the integrase coding sequences. The integrase was excluded from all of the constructs described here to avoid possible integration of vaccine sequences into the host genome. To improve Pol expression, the frameshift region between the *gag* and *pol* genes was mutated by single-base insertion to create gagpol with both the Gag and Pol coding sequences in the same open reading frame. Creation of this

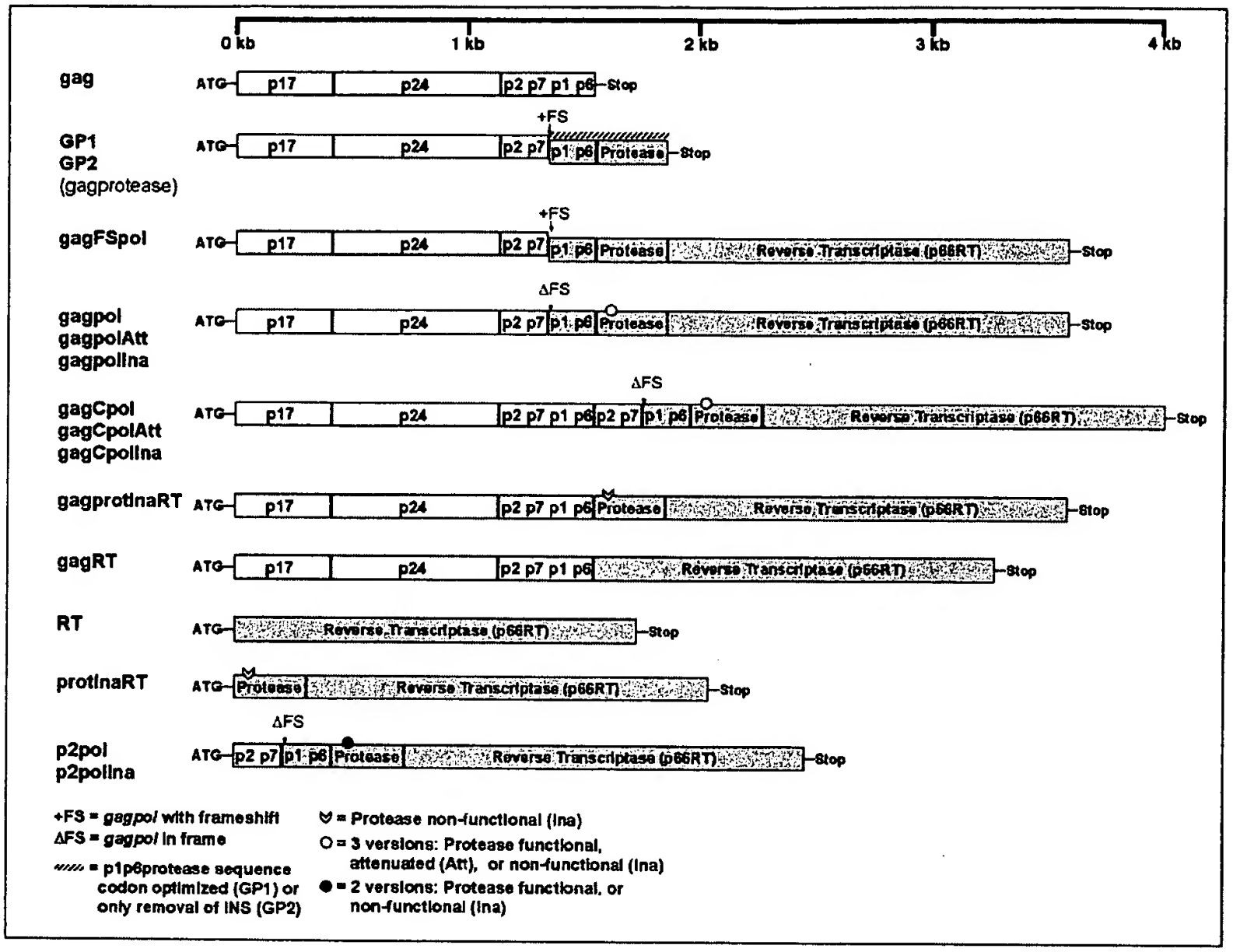

FIG. 1. Overview of HIV-1 *gag* and *pol* expression cassettes. All sequences are based on the HIV-1_{SF2} isolate (GenBank accession no. K02007) and were optimized for human codon usage. The coding sequence for RT was mutated for all affected constructs to yield a nonfunctional protein. The various versions of constructs with mutations to eliminate the frameshift (FS) and protease (Prot) activity are shown.

construct resulted in the loss of $p1p6^{Gag}$ because of the mutation introduced to remove the frameshift. Because the p6 portion of Gag was shown to be important for the efficient release of Gag VLP (19), the cassette gagCpol was designed to include a repeat of p2p7p1p6 to restore p1p6Gag expression. Moreover, the $p2p7^{Gag}$ repeat was introduced to improve the secretion and autoprocessing of gagCpol by the protease (1, 57). Also, to enhance possible processing requirements for efficient expression, a *pol* cassette was designed to include *p2p7gag* (p2pol and p2pol). Because of concerns regarding potential cytotoxic properties of the functional viral protease (32) that could affect both antigen expression and immunogenicity, the *protease* gene was either attenuated (Att) or rendered inactive (Ina) in the designated constructs (Fig. 1). Fusion cassettes expressing Gag plus RT (gagRT) and Gag plus protease plus RT (gagprotInaRT) were also constructed and compared to gagCpol.

In vitro characterization of expression cassettes. To evaluate the expression patterns of the various Gag- and Pol-containing constructs, 293 cells were transiently transfected and supernatants and cell lysates were analyzed by $p24^{Gag}$ antigen capture ELISA and immunoblotting. Because the $p24^{Gag}$ antigen capture ELISA preferentially recognizes processed forms of Gag (48, 56), comparative expression analyses were problematic to perform for all constructs. However, comparison of very similar constructs allowed us to test for differences in Gag expression.

Figure 2 illustrates the relative Gag expression levels. The cassette gagFSpol was designed to extend the Pol region and at the same time maintain the natural processing and frameshift translation of the expressed GagPol precursor polyprotein. In cell lysates, the expression level of Gag from this construct was about the same as that of Gag expressed by GP2 (Fig. 2B) but about fourfold less $p24^{Gag}$ was detected in the culture supernatant compared to that of GP2 (Fig. 2A). In the gagpol and gagCpol constructs, the frameshift sequences were altered so that Gag and Pol could be expressed by the same reading frame in order to increase the expression of Pol without affecting Gag expression. In alternative versions of these constructs, the *protease* gene was either mutated to produce attenuated (gagpolAtt, gagCpolAtt) or inactivated (gagpolIna, gagCpolIna) protease. As shown in Fig. 2C, no differences in

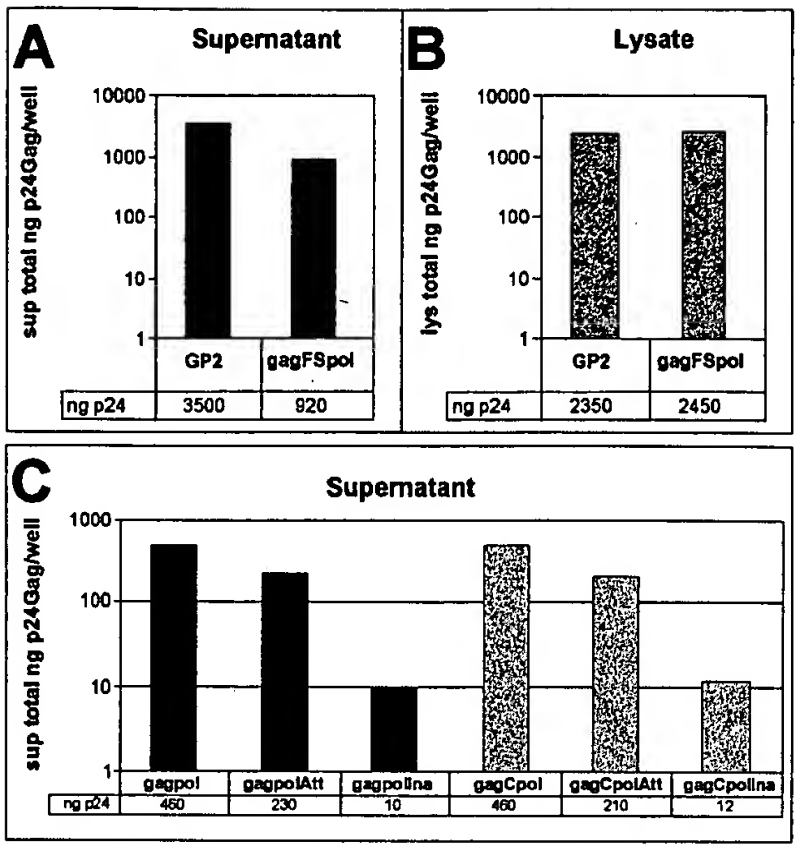

FIG. 2. Quantitative comparison of HIV-1 Gag expression by $p24^{Gag}$ antigen capture ELISA of supernatants (sup) and lysates (lys) of 293 cells 48 h posttransfection with *gagpol* constructs. Supernatants (A) and lysates (B) of GP2 versus gagFSpol are shown. Both cassettes express functional protease with an intact frameshift. In the experiment whose results are shown in panel C, supernatants were analyzed from different versions of in-frame gagpol versus gagCpol with functional, nonfunctional, or attenuated protease. The gagCpol cassettes included an additional *p2p7p1p6*^{gag} sequence.

$p24^{Gag}$ levels were observed in culture supernatants when similar versions of gagpol and gagCpol were compared. The same results were also obtained with the cell lysates (data not shown). Thus, the additional insertion of the *p2p7p1p6* fragment appeared to have no influence on $p24^{Gag}$ expression levels as measured here.

Western blot analysis was performed with all of the expression cassettes described in Fig. 1 by using Gag-specific, HIV-positive human antisera (Fig. 3A and B). Clear differences were observed between plasmids expressing processed and unprocessed forms of the Gag and GagPol polyproteins. The highest level of Gag-specific reactivity appeared to be found in supernatants (Fig. 3A) and lysates (Fig. 3B) of cultures of cells transfected with gag, followed by GP2 and gagFSpol (data not shown). GP2 and gagFSpol process the Gag polyprotein by using a protease that is underexpressed with the natural frameshift intact, and the bands observed included unprocessed $p55^{Gag}$ and processed forms of Gag. As would be expected in the absence of protease, very little or no processed $p24^{Gag}$ was seen in lysates of cells expressing Gag alone; nevertheless, the small amount of processing observed in the supernatants of these cells was likely due to the presence of nonspecific cellular protease activity. In transfections with two of the constructs expressing Gag and Pol in the same reading frame, gagCpol and gagCpolAtt, the band corresponding to $p55^{Gag}$ was not detectable in the cell supernatants or lysates and reduced amounts of $p24^{Gag}$ were seen in supernatants and lysates (Fig. 3A and B). For gagCpolIna with the nonfunctional protease, no Gag-specific bands were detected in cell supernatants (Fig. 3A) and a high-molecular-mass band corresponding to the unprocessed GagCPol polyprotein (149 kDa) was observed to migrate as expected in the cell lysate (Fig. 3B). Additional bands expressed from gagCpolIna included small amounts of $p55^{Gag}$ and $p41^{Gag}$, but no $p24^{Gag}$ could be detected. Accordingly, when cells transfected with gagCpolIna, gagCpol, and gag were examined by electron microscopy, very few VLP were detected for gagCpolIna and no particles were detected for gagCpol, indicating impaired secretion of VLP compared to that achieved with gag (data not shown). The cassettes gagRT (121 kDa) and gagprotInaRT (131 kDa) showed levels of Gag comparable to those observed for gagCpolIna (data not shown).

The expression of Pol in cell lysates from transfected 293 cells was also analyzed by Western blotting with RT-specific antisera (Fig. 3C). In general, both the single-gene cassettes in the absence of Gag (RT, proteaseRT, and p2polIna) and the *gagpol* fusion cassettes (gagRT, gagprotInaRT, gagCpolIna, and gagpolIna) appeared to be expressed well as long as the *protease* gene was absent or nonfunctional. The RT (66 kDa) and protInaRT (75 kDa) cassettes appeared to be expressed at the highest levels, followed by the p2polIna (93 kDa), gagRT (121 kDa), and gagprotInaRT (131 kDa) cassettes, followed by the gagCpolIna (149 kDa) and gagpolIna (132 kDa) cassettes. The latter two constructs exhibited high-molecular-weight bands of the expected relative mobilities (and slightly faster, respectively) of similar intensities indicative of comparable levels of expression. In constructs expressing the functional and attenuated HIV protease, p2pol, gagCpol, and gagCpolAtt, reduced expression of RT-specific bands was observed compared to the levels expressed by the p2polIna and gagCpolIna constructs. In summary, the addition of *gag* sequences to *pol* appeared to have very little influence on Pol-specific expression levels and vice versa but the addition of a functional *protease* gene resulted in reduced expression of Gag- and RT-specific bands.

Design of mouse immunogenicity studies. The relative immunogenicities of the DNA plasmids encoding the various gene cassettes were evaluated in mice that were intramuscularly immunized with doses of plasmid DNA ranging from 0.002 to 20 μg (Table 1 contains a summary of the studies performed). This afforded a determination of the dose dependency for each plasmid. In each experiment, groups of 4 to 10 mice were immunized per dose of a given plasmid. One set of mice was immunized twice, at weeks 0 and 4, with spleen removal and analysis at week 6, and another set was immunized once with DNA and then challenged after 4 weeks with rVV expressing GagPol or Pol. Spleens were removed 5 days later, and cells were harvested for ICS to measure Gag- and Pol-specific IFN-γ-producing $CD8^+$ lymphocytes. Because boosting with rVV enhanced specific immune responses to these antigens, T-cell responses could be evaluated after a single DNA prime even at the lowest DNA dose.

$CD8^+$ T-cell responses to Gag. Gag-specific $CD8^+$ T-cell responses were analyzed by intracellular IFN-γ staining of $CD8^+$ spleen cells that had been stimulated with Gag peptide p7g, an H-$2K^d$-restricted epitope (Doe and Walker, letter). In

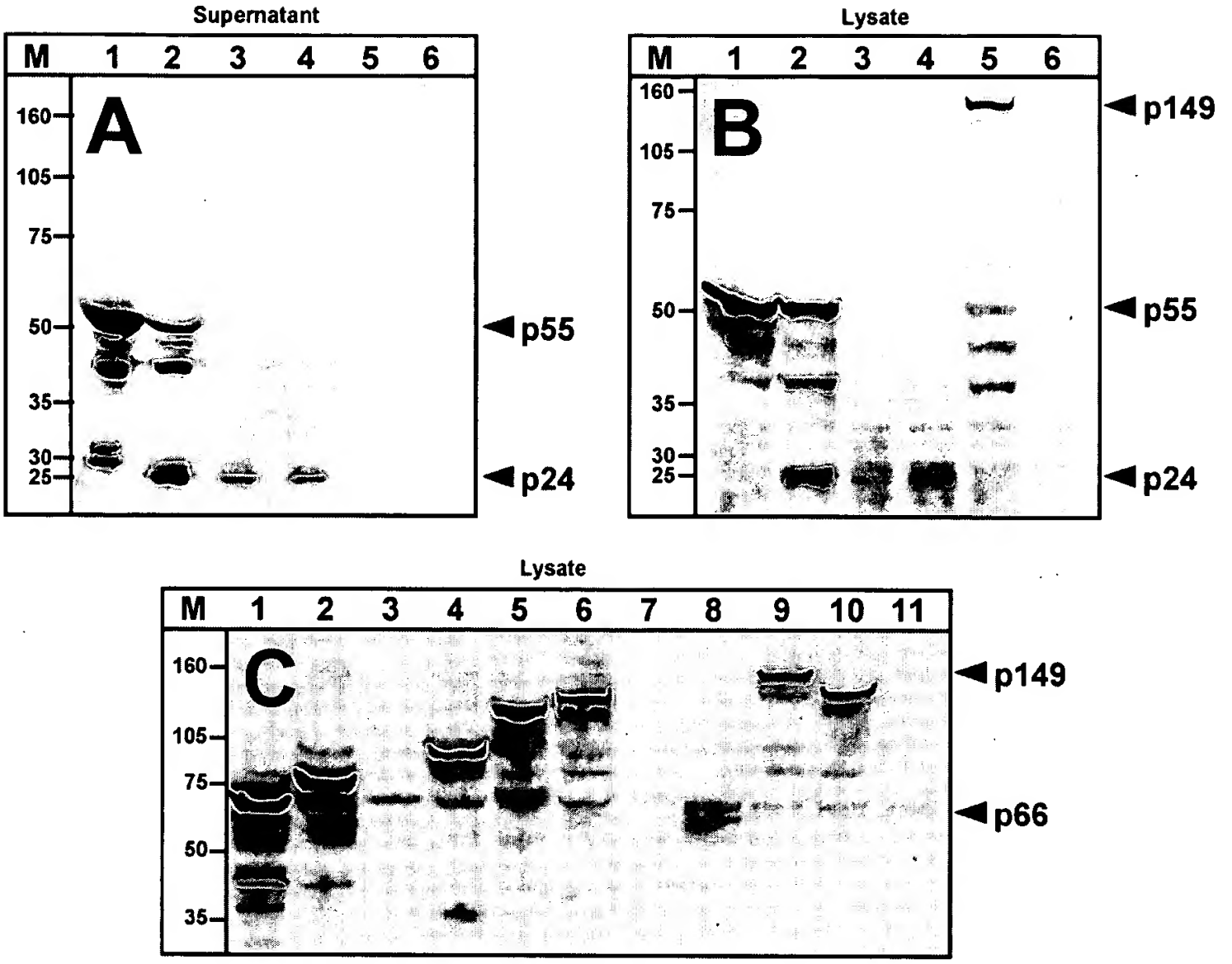

FIG. 3. Immunoblots of synthetic HIV-1 *gag* and *pol* expression cassettes. 293 cells were transfected, and supernatants and lysates were collected 48 h posttransfection, subjected to 4 to 12% Bis-Tris sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and blotted onto nitrocellulose membranes. Immunostaining was performed with either human HIV-1 patient serum (A and B) or pooled anti-$p66^{RT}$ mouse serum (C). For detection of Gag expression, supernatants (A) and lysates (B) were used. Lanes: 1, gag; 2, GP2; 3, gagCpol; 4, gagCpolAtt; 5, gagCpolIna; 6, mock transfection. For detection of Pol products, only cell lysates were analyzed (C). Lanes: 1, RT; 2, protInaRT; 3, p2pol; 4, p2polIna; 5, gagRT; 6, gagprotInaRT; 7, gagCpol; 8, gagCpolAtt; 9, gagCpolIna; 10, gagpolIna; 11, mock transfection. The values on the left are molecular sizes (M) in kilodaltons.

the first study (Fig. 4), addition of functional protease to Gag with a frameshift in constructs GP1 and GP2 was tested. The $CD8^+$ T-cell responses after two DNA immunizations were indistinguishable for all three plasmids. Thus, from these results, protease-mediated cleavage of Gag apparently did not affect the processing and presentation of Gag in vivo. At the lowest plasmid dose (0.02 μg), Gag-specific $CD8^+$ T cells were only 30 to 50% below maximum. Therefore, for the next studies, the lowest DNA dose was reduced further to 0.002 μg. Furthermore, new constructs were included and compared to *gag*. The potency of all of the plasmids tested with regard to the induction of Gag-specific $CD8^+$ T cells was indistinguishable after a single DNA immunization followed by an rVVgagpol challenge or after two DNA immunizations (Fig. 5A and B).

Addition of *pol* sequences to *gag* in the DNA vaccine constructs evaluated here did not affect the induction of Gag-specific immune responses. Moreover, despite apparent differences between gagCpol and gagCpolIna in Gag expression as measured in vitro (Fig. 3), the induction of Gag-specific $CD8^+$ T-cell responses was not affected by functional protease.

Antibody responses to Gag. The measurement of Gag-specific antibody responses revealed a different pattern of responses for the various constructs compared to that observed for the cellular responses. In the first experiment, a comparison was drawn between gag and GP1 and GP2 (Fig. 6A) to look for possible effects of the functional protease on the immunogenicity of $p55^{Gag}$ when protease is expressed with the natural frameshift. The $p55^{Gag}$ antibody responses at 2 weeks post second DNA immunization demonstrated the overall weakest responses with GP1 and better responses with GP2. The gag DNA appeared to be more immunogenic, especially at the lower DNA doses, but GP2 was more comparable to gag at the highest DNA dose (20 μg). For the next experiment (Fig. 6B), antibody responses were analyzed 5 days after a vaccinia virus challenge. Additional cassettes expressing Gag and Pol in frame (gagprotInaRT, gagCpol, and gagCpolIna) were evaluated. In comparison to the previously described analysis (Fig. 6A), the differences between constructs were much more apparent. Two patterns of antibody induction emerged. The gag and gagFSpol cassettes induced strong humoral immune re-

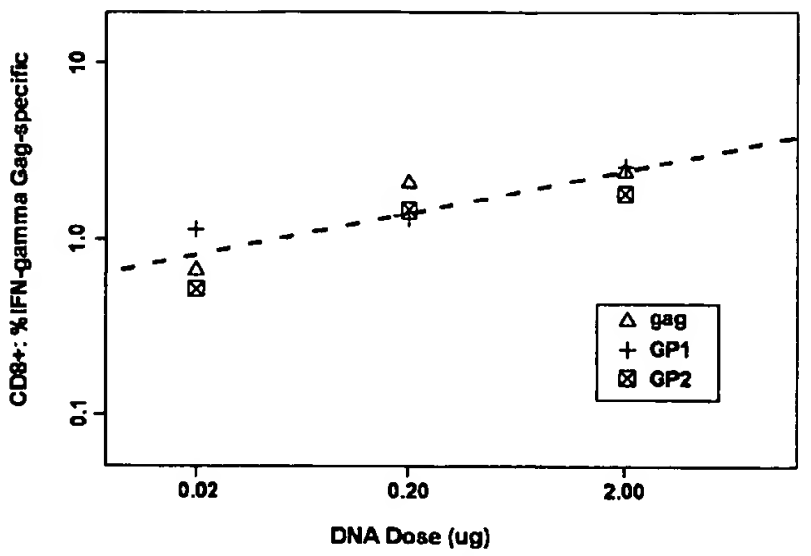

FIG. 4. Quantitative analysis of Gag-specific, IFN-γ-secreting $CD8^+$ T cells. CB6F1 mice were immunized twice at weeks 0 and 4 with titrated doses of codon-optimized $HIV\text{-}1_{SF2}$ gag, GP1, and GP2 plasmid DNAs. Spleens were removed 2 weeks after the second immunization, and the pooled spleen cells were stimulated in duplicate for 5 h with the p7g peptide. On the following day, cells were stained for $CD8^+$ and intracellular IFN-γ and analyzed by flow cytometry. Data were analyzed by a regression model (see Materials and Methods for details).

sponses post vaccinia virus challenge, while the gagprotInaRT, gagCpol, and gagCpolIna cassettes were much less potent for the induction of antibody titers. The observed antibody responses appear to correlate with the relative amounts of secreted Gag proteins observed in the in vitro analysis (Fig. 3). The constructs that secreted the highest levels of Gag (gag and gagFSpol) primed for the most potent antibody responses, while those that expressed high-molecular-weight polyproteins in the cell lysates (gagprotInaRT and gagCpolIna) or overprocessed Gag (gagCpol) induced the poorest antibody responses.

Cellular immune responses to Pol. For detection of cellular immune responses to Pol, studies were done with C3H/HeN mice. Spleen cells were stimulated with the $H\text{-}2K^k$-restricted nonamer TEMEKGEKI (35) and analyzed by flow cytometry for IFN-γ synthesis. Figure 7A and C compare the RT and protInaRT DNA vaccines with those encoding *gag* plus *pol* sequences. In general, the magnitude of the Pol responses was lower than that of the Gag responses. No significant differences were observed between the different antigens, with the exception of gagFSpol, which was not as potent as expected as a result of the low-level expression of the encoded Pol products. Figure 7B and D show that the p2pol cassette, in which the *p2p7gag* and *p1p6pol* sequences precede protInaRT, induced Pol-specific $CD8^+$ T cells, even at low doses. Thus, in-frame insertions of *p2p7p1p6* and *protease* upstream of *RT* did not seem to reduce RT-specific immunogenicity. To study this further, the complete *gag* coding region was inserted upstream of *pol*. As shown in Fig. 7, in-frame insertion of *gag* did not suppress the induction of RT-specific $CD8^+$ T cells; however, if the wild-type frameshift was present (gagFSpol), the vaccine was less potent at inducing this Pol-specific response after a vaccinia virus boost for all doses (Fig. 7B) and no response was detectable after two DNA immunizations, even at the highest dose (Fig. 7D). As for immune responses to Gag, the differences in Pol expression in gagCpol constructs with functional and nonfunctional protease, as seen in vitro, did not result in differences in the observed immune potencies of these constructs. The cellular immune responses to Pol, as measured here, were not affected either by the activity of protease or by the addition of *gag* sequences upstream of *pol*.

DISCUSSION

For the design and development of an effective HIV-1 vaccine, the induction of T-cell responses with a large repertoire of specificities is essential. Inclusion of HIV-1 Pol in a vaccine would be expected to increase this repertoire significantly (54). Pol is well-conserved, broad CTL responses are found in the majority of infected patients, and these responses have been shown to be inversely correlated to the viral load (7, 21). Since the virus-encoded *pol* gene is expressed at very low levels compared to *gag* as a result of the translational frameshifting mechanism by which it is expressed, increasing *pol* expression

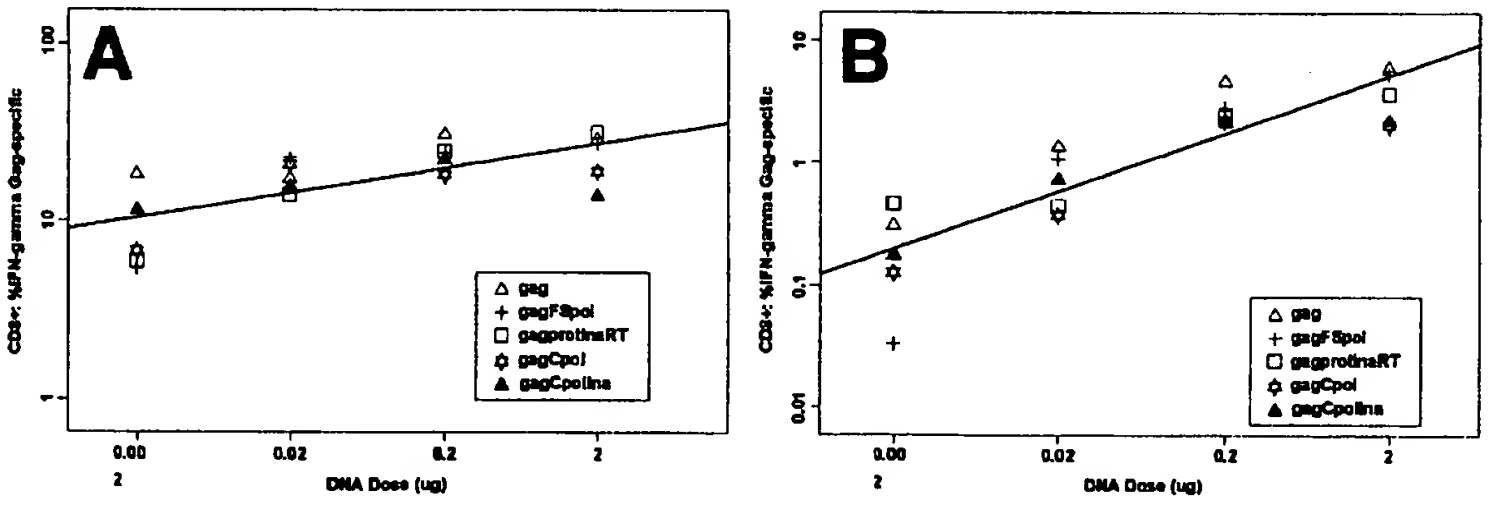

FIG. 5. $HIV\text{-}1_{SF2}$ Gag-specific $CD8^+$ responses of CB6F1 mice immunized with titrated DNA doses of *gag* or *gag*-plus-*pol* cassettes. Groups of mice were either immunized once and challenged with rVVgagpol 4 weeks later (A) or received two immunizations with DNA at weeks 0 and 4 (B). Spleens were harvested 5 days post vaccinia virus challenge or 2 weeks post second immunization, respectively. Pooled splenocytes were stimulated with the Gag-specific peptide p7g for 5 h. Cells were stained for $CD8^+$ and intracellular IFN-γ on the next day and analyzed by flow cytometry. Data were analyzed by a regression model (see Materials and Methods for details).

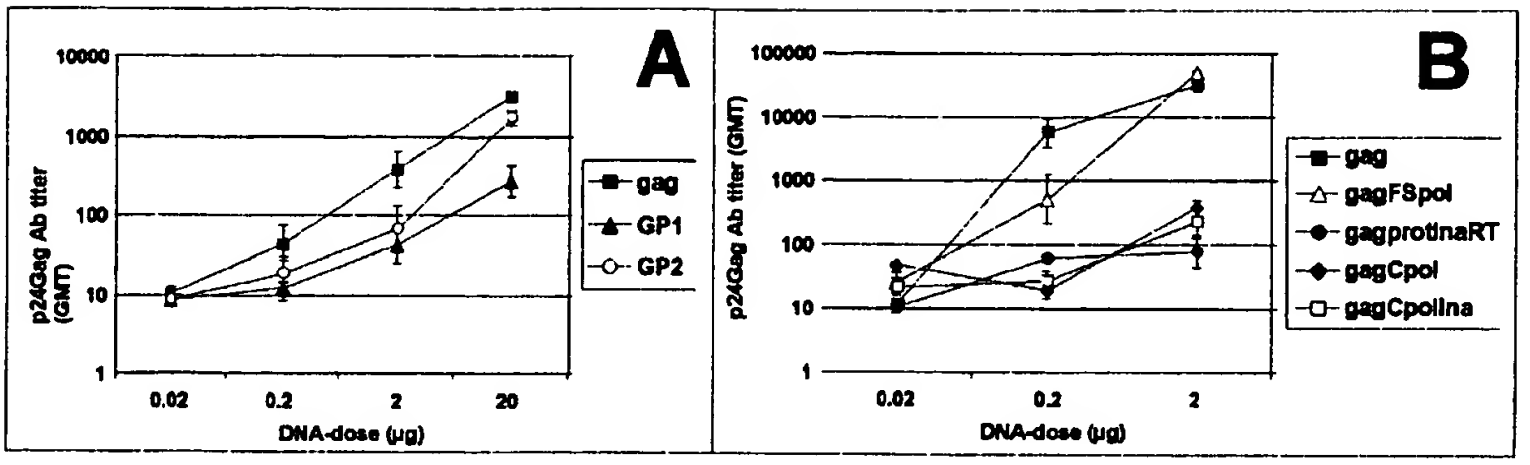

FIG. 6. Antibody (Ab) titers specific for HIV-1_{SF2} $p24^{Gag}$ in mice 2 weeks after two immunizations (weeks 0 and 4) with DNA (A) or 5 days postchallenge with rVVgagpol after a single DNA immunization at week 0 (B). Collected serum samples were analyzed by $p24^{Gag}$ ELISA as described in Materials and Methods. (A) The plasmid expressing only $p55^{Gag}$ (gag) was compared to GP1 and GP2. (B) Expression cassettes gag and gagFSpol were compared to nonframeshifted versions of gagpol. The values shown are the geometric mean antibody titers and the standard deviations of the midpoint antibody titers for each group.

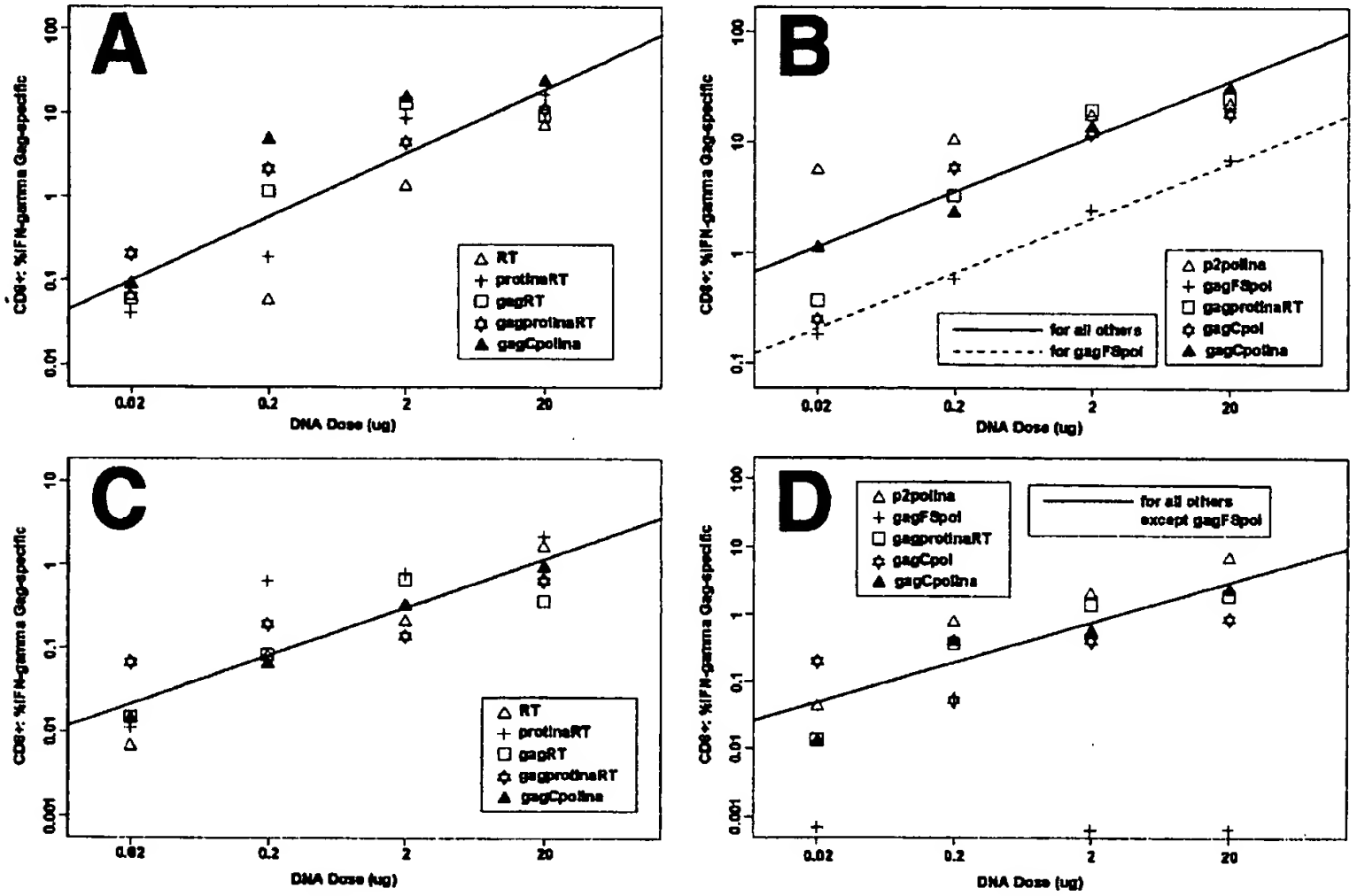

FIG. 7. Frequencies of HIV-1_{SF2} RT-specific $CD8^+$ T-cell responses of C3H/HeN mice immunized with titrated DNA doses of RT, protInaRT, p2polIna, or *gag*-plus-*pol* cassettes. Results for mice immunized once and challenged with rVVpol 4 weeks later are shown in panels A and B. Another set of animals received two DNA immunizations at weeks 0 and 4 (C and D). Spleen harvesting and staining for flow cytometry were performed as described in the legend to Fig. 5. Data were analyzed by a regression model (see Materials and Methods for details).

by removal of the natural frameshift and removal of inhibitory sequences could result in the induction of a higher frequency of Pol-specific effector and memory CTL by *pol*-based DNA vaccines. In addition, because an effective HIV-1 vaccine would very likely be composed of at least *gag* and *pol* plus *env*, cost and practicability should also be considered. A multigenic DNA vaccine containing *gag* and *pol* on one plasmid would therefore be an advantage. Gene cassettes encoding *gagpol* have been used previously in vaccines with modest immunological outcomes with respect to the induction of Pol-specific T-cell responses in human and nonhuman primate studies (9, 15, 16). This could be explained by the use of the *gagpol* gene

with an intact frameshift and/or native codon usage, which would be expected to provide lower levels of *pol* expression. Casimiro et al. reported recently for the first time strong Pol-specific cellular immune responses in nonhuman primates after immunization with synthetic *pol* DNA vaccines (10).

In this work, we analyzed immune responses to HIV-1 *gag* and a variety of *pol* sequences in separate and combined expression cassettes. Particular attention was given to the possible negative effect of *pol* on *gag* expression and immunogenicity. Immune responses to the well-characterized plasmid pCMVKm2.GagMod.SF2 (gag) (56) served as a benchmark for these studies. Results obtained with the sequence-modified *pol* gene indicated that the expression and immunogenicity of Gag using gagFSpol with an intact frameshift was not affected by the *pol* sequence (Fig. 5 and 6B). Also, after removal of the frameshift region from the *gagpol* cassettes, Pol expression was improved dramatically. While Pol expression could not be detected in Western blots of lysates and culture supernatants from cells transfected with gagFSpol (data not shown), plasmids encoding an in-frame *gagpol* cassette with nonfunctional protease showed high-level expression (Fig. 3C). This was also confirmed in mice immunized with gagFSpol versus gagCpol in-frame cassettes. Pol-specific $CD8^+$ T-cell responses could only be detected in gagFSpol-immunized animals after an rVVpol boost, whereas gagCpol induced strong responses after two DNA doses (Fig. 7B and D). Interestingly, previously described cytotoxic effects of HIV-1 protease that were shown to affect the expression of additional genes in vivo (51) did not diminish $CD8^+$ T-cell responses. The gagCpol (functional protease) and gagCpolIna (nonfunctional protease) DNA vaccines were indistinguishable in their abilities to induce cellular immune responses to Gag or Pol (Fig. 5 and 7B and D). However, reduced expression of the Gag and Pol proteins was observed in Western blots of transfected cells when the protease was functional (Fig. 3). Whether this effect was directly related to negative effects of protease or altered expression kinetics remains to be determined.

HIV-1 Gag is a major target with respect to the induction of CTL responses in HIV-1-infected patients, and $p24^{Gag}$ and $p17^{Gag}$ appear to have the highest epitope density, besides Nef, of all HIV-1 antigens (55). Recently, an important contribution of $p15^{Gag}$ to the overall CTL response in HIV-1-infected subjects also was reported (55). This result should be considered in a Gag-based vaccine design. Thus, to retain important epitopes for Gag, the gagCpol cassette, containing the complete *gag* coding sequences in addition to *pol* in frame, was designed. After removal of the frameshift by a single-base insertion, $p1p6^{Gag}$ protein expression was lost, resulting in a truncated Gag protein that was shortened by $p1p6^{Gag}$ at the frameshift site. The extension of gagpol to include $p2p7p1p6^{Gag}$ in the gagCpol construct had no negative influence on expression (Fig. 2C), and this cassette design was therefore selected for use in immunogenicity studies instead of the original gagpol construct.

Immune responses generated against Gag or Pol by using various Gag- and Pol-expressing DNA vaccines were evaluated by repeated experiments with either two DNA immunizations or one immunization followed by an rVV boost. Responses were scored by flow cytometric measurements of antigen-specific IFN-γ-secreting $CD8^+$ cells with an ICS assay. Responses to Gag were detectable after two immunizations with amounts of DNA as small as 2 ng. No significant differences in Gag-specific $CD8^+$ T-cell responses were found for any of the sequence-modified expression cassettes tested here. Cellular immune responses to Pol were analyzed by using C3H mice ($H\text{-}2^k$), and spleen cells were stimulated by using the 9-mer CTL peptide described by Hosmalin et al. (25). Positive responses could be detected in the 20- to 200-ng DNA dose range, compared to 2 ng for Gag. This could be explained by the reduced recognition and assay sensitivity of this peptide as recently described (10). However, solid stimulation was demonstrated with this peptide epitope; up to 32% of RT-specific $CD8^+$ cells responded after one 20-μg DNA prime and an rVV boost (Fig. 7A and B). As expected from the expression results, the gagFSpol DNA vaccine (i.e., Pol expressed with a frameshift) induced significantly lower levels of Pol-specific immune responses if DNA-primed mice were boosted with rVV expressing Pol (Fig. 7B) and no detectable Pol-specific responses after two DNA immunizations (Fig. 7D). As for Gag responses, no significant differences were found among the in-frame sequence-modified constructs with regard to the induction of Pol-specific $CD8^+$ T-cell responses. Thus, it appears that efficient secretion of Gag antigens as VLP secretion, which is impaired in gagpol fusion constructs (28, 40), was not essential for the induction of potent Gag-specific $CD8^+$ responses. Previous results obtained by another group using synthetic *pol* and *gagpol* genes also demonstrated improved expression of Pol when it was fused in frame with Gag (26). However, cellular immune responses to Gag and Pol were demonstrated for single and fusion gene cassettes when mice were immunized four times with 100 μg of DNA. In our experiments, we titrated the DNA doses down to 2 ng for Gag responses and 20 ng for Pol responses, which allowed us to more fully evaluate the relative potency of each construct. Moreover, in the present study, several additional versions of *pol* and *gagpol*, including those with an attenuated, functional and nonfunctional protease gene, were analyzed.

Altogether, the data presented in this study suggest that the highly efficient expression and immunogenicity of Gag are not impaired by Pol, and vice versa, if Gag and Pol are expressed as a multigenic fusion protein (gagCpol) in a DNA vaccine. Moreover, the expression and immunogenicity of the Pol antigen can be enhanced through removal of the frameshift and sequence modifications to remove inhibitory sequences and optimize codon usage. The improved *gag*-plus-*pol* DNA vaccine described here, when administered by using recently described enhanced DNA vaccine delivery technologies (38, 52), should prove to be a potent vaccine for the induction of T-cell immune responses. Furthermore, vaccine approaches that combine the gagCpol DNA vaccine for the induction of cellular immune responses with improved Env antigens for the induction of neutralizing antibodies (3, 13, 50) hold great promise for the next generation of HIV vaccines.

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Anti-HIV env immunities elicited by nucleic acid vaccines.

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Plasmid DNA vaccines encoding HIV-1 env were used to immunize mice and nonhuman primates. Plasmids were prepared that produced either secreted gp120 or full-length gp160. Mice immunized with gp120 DNA developed strong antigen-specific antibody responses, CD8+ cytotoxic T lymphocytes (CTL) (following in vitro restimulation with gp120-derived peptide), and showed in vitro proliferation and Th1-like cytokine secretion [gamma-interferon, interleukin (IL)-2 with little or no IL-4] by lymphocytes obtained from all lymphatic compartments tested (spleen, blood, and inguinal, iliac, and mesenteric lymph nodes). This indicated that systemic anti-gp120 cell-mediated immunity was induced by this DNA vaccine. Although similar antibody responses were observed in mice immunized by either intramuscular or intradermal routes, T cell responses were significantly stronger in mice injected intramuscularly. Rhesus monkeys immunized with both gp120 and gp160 DNAs exhibited significant CD8+ CTL responses, following in vitro restimulation of peripheral blood lymphocytes with antigen. These experiments demonstrate that DNA immunization elicits potent immune responses against HIV env in both a rodent and a nonhuman primate species.

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J Immunol Methods. 1998 Nov 1;220(1-2):93-103. Related Articles, Links

Intranasal immunization with a plant virus expressing a peptide from HIV-1 gp41 stimulates better mucosal and systemic HIV-1-specific IgA and IgG than ral immunization.

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Control of pandemic human immunodeficiency virus type 1 (HIV-1) infection ideally requires specific mucosal immunity to protect the genital regions through which transmission more often occurs. Thus a vaccine that stimulates a disseminated mucosal and systemic protective immune response would be extremely useful. Here we have investigated the ability of a chimeric plant virus, cowpea mosaic virus (CPMV), expressing a 22 amino acid peptide (residues 731-752) of the transmembrane gp41 protein of HIV-1 IIIB (CPMV-HIV/1), to stimulate HIV-1-specific and CPMV-specific mucosal and serum antibody following intranasal or oral immunization together with the widely used mucosal adjuvant, cholera toxin. CPMV-HIV/1 has been shown previously to stimulate HIV-1-specific serum antibody in mice by parenteral immunization. All mice immunized intranasally with two doses of 10 microg of CPMV-HIV/1 produced both HIV-1-specific IgA in faeces as well as higher levels of specific, predominantly IgG2a, serum antibody. Thus there was a predominantly T helper 1 cell response. All mice also responded strongly to CPMV epitopes. Oral immunization of the chimeric cowpea mosaic virus was less effective, even at doses of 500 microg or greater, and stimulated HIV-1-specific serum antibody in only a minority of mice, and no faecal HIV-1 specific IgA.

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Scheme for Ranking Potential HLA-A2 Binding Peptides Based on Independent Binding of Individual Peptide Side-Chains

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Abstract. A method to predict the relative binding strengths of all possible nonapeptides to the MHC class I molecule HLA-A2 has been developed based on experimental peptide binding data. These data indicate that, for most peptides, each side-chain of the peptide contributes a certain amount to the stability of the HLA-A2 complex that is independent of the sequence of the peptide. To quantify these contributions, the binding data from a set of 154 peptides were combined together to generate a table containing 180 coefficients (20 amino acids × 9 positions), each of which represents the contribution of one particular amino acid residue at a specified position within the peptide to binding to HLA-A2. Eighty peptides formed stable HLA-A2 complexes, as assessed by measuring the rate of dissociation of β_2m. The remaining 74 peptides formed complexes that had a half-life of β_2m dissociation of less than 5 min at 37°C, or did not bind to HLA-A2, and were included because they could be used to constrain the values of some of the coefficients. The "theoretical" binding stability (calculated by multiplying together the corresponding coefficients) matched the experimental binding stability to within a factor of 5. The coefficients were then used to calculate the theoretical binding stability for all the previously identified self or antigenic nonamer peptides known to bind to HLA-A2. The binding stability for all other nonamer peptides that could be generated from the proteins from which these peptides were derived was also predicted. In every case, the previously described HLA-A2 binding peptides were ranked in the top 2% of all possible nonamers for each source protein. Therefore, most biologically relevant nonamer peptides should be identifiable using the table of coefficients. We conclude that the side-chains of most nonamer peptides to the first approximation bind independently of one another to the HLA-A2 molecule. *Journal of Immunology,* 1994, 152: 163.

MHC class I molecules are normally expressed on the cell surface in a stable complex, with any one of a large number of peptides generated upon proteolysis of intracellular proteins (1, 2). In theory, each allelic variant of a class I MHC molecule selects these peptides based on the complementary structure of the peptide and the polymorphic pockets within the peptide-binding groove (3, 4). In the past, motifs specific to individual class I molecules have been determined by comparing the sequences of endogenous peptides isolated from purified class I molecules (5, 6), or by comparing the sequences of peptides that are known to bind to each class I molecule (7). In every case studied so far, at certain positions within the peptide, one aa[2] or a small number of related aa are found to be nearly invariant; these aa are called dominant anchor residues (5) and appear to "anchor" the peptide into the class I peptide binding site by having a structure that is complementary to a pocket of the peptide-binding groove. For example, endogenous peptides isolated from purified HLA-A2 contain as dominant anchor residues Leu or Met at P2, and Val or Leu at P9 (5, 8), which are thought to bind in the B and F pockets, respectively (4). Some of the other positions within the endogenous peptides are also enriched for specific aa; these are defined as auxiliary anchor residues (5). In many cases, it is not clear to what degree the allele-specific peptide-binding motifs consisting of dominant and auxiliary anchor residues are a consequence of the requirements

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[2] Abbreviations used in this paper: aa, amino acid residue; GF, gel filtration; IBS, independent binding of side-chains; M1, influenza A matrix protein peptide 58–66 (GILGFVFTL); P1, position 1 in a peptide; PΩ, last position in a peptide; β_2m, β_2-microglobulin.

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for peptide binding (9), or whether part of the motif is a consequence of Ag processing (5). These motifs have occasionally proven useful for localizing the optimal class I binding peptide within the sequence of a longer peptide that is known to contain a HLA-A2-restricted T cell epitope (10, 11). However, in other cases, no obvious motif is present within the antigenic peptide (12–15), possibly reflecting the limitations in our knowledge about what are acceptable variations in peptide-binding motifs.

To determine to what degree the dominant anchor residues or the auxiliary anchor residues of the HLA-A2 motif are important for peptide binding, we have extended our previous peptide-binding study (16) to include a more extensive set of nonapeptides, many of which are interrelated by single aa substitutions. These new data indicate that at each position within the peptide, some aa are more favorable than others, regardless of the sequence of the rest of the peptide; therefore, it should theoretically be possible to improve upon predictions based solely on the anchor residues at P2 and P9 that are specific to HLA-A2 binding peptides. We present a table of 180 coefficients specific for each of the 20 aa at each of the 9 positions within the peptide. This table of coefficients incorporates all of the data that we have collected and can be used to predict the stability of HLA-A2 complexes containing any desired peptide. A mathematical combinatorial approach similar to that used to generate the HLA-A2 binding coefficients could also be applied to other macromolecular interactions, such as between oligonucleotides and DNA-binding proteins.

Materials and Methods

Peptides

Peptides were synthesized and purified as described (17).

HLA-A2 binding assays

Native isoelectric focusing gel and GF peptide binding assays were used as described (17). These assays measure peptide binding indirectly by monitoring the ability of peptides to promote incorporation of ^{125}I-labeled β_2m into HLA-A2/β_2m/peptide heterotrimeric complexes. Because the HLA H chain is refolded from inclusion bodies prepared from *Escherichia coli*, there are no endogenous peptides present to confound the data. Instead, ^{125}I-β_2m is incorporated into HLA complexes only when an appropriate synthetic peptide is present.

Rate measurements

The stability of HLA-A2 complexes containing specific peptides was assessed by measuring the β_2m dissociation rate as described (17). Briefly, HLA complexes containing ^{125}I-β_2m were isolated by GF and β_2m dissociation was measured by means of a second round of GF. To make accurate measurements of half-lives that were less than 20 min, it was necessary to collect the purified complexes from the first round of GF directly into microcentrifuge tubes that were then maintained at 0°C. Each aliquot to be used for a time point was separately incubated from between 1 and 30 min at 37°C before the second round of GF.

Mathematical modeling

To combine the data mathematically from a large number of experiments, a Fortran program was written that could optimize for the values of all 180 (20 aa × 9 positions) coefficients with any number of simultaneous equations. The rate data for a peptide whose sequence was GILGFVFTL would be entered as follows:

$$\mathrm{ERR} = \ln(t_{1/2}) - \ln(\mathrm{G1} \times \mathrm{I2} \times \mathrm{L3} \times \mathrm{G4} \times \mathrm{F5} \times \mathrm{V6} \times \mathrm{F7} \times \mathrm{T8} \times \mathrm{L9} \times \mathrm{Constant})$$

where ERR squared equals the error function to be minimized, $t_{1/2}$ equals the measured half-life of dissociation in minutes at 37°C, G1 represents, for example, the coefficient for Gly at P1 to be determined (see Table I), and Constant equals the overall normalization constant. For the peptides that had half-lives of dissociation of <5 min, ERR was set equal to 0.0 if the second term (the logarithm of the product of the coefficients and the normalization constant) was less than ln(5.0); otherwise, ln(5.0) was subtracted from the second term. The program calculated the values for the coefficients that minimized the sum of the error functions of the 154 equations, where each equation corresponds to the rate data for one peptide. After the coefficients were calculated, the coefficients at each position were normalized by dividing by the coefficient for Ala at that same position, and all of the normalization constants for Ala at each position were combined into Constant. As a result, the coefficients for Ala are not independent, and there are only 172 independent variables: one for each aa (19 independent terms relative to Ala) at each of the 9 positions within a nonameric peptide, plus Constant. Thus, Constant equals the predicted half-time for dissociation of a complex containing the peptide AAAAAAAAA in minutes at 37°C.

In practice, to limit the number of variables to a number more appropriate for the amount of data we had collected, 82 variables were selected that appear to be the most important for determining peptide binding to HLA-A2 (see Table V). Of these variable coefficients 40 were at the dominant anchor positions at P2 and P9, which are known to be critical for peptide binding to HLA-A2 (5, 8). A total of 31 variable coefficients were selected on the basis of the relationship between the sequences of the peptides and the data that were collected. For example, a coefficient was always allowed to be a variable if the dissociation rate for an HLA-A2 complex containing a peptide was significantly different from the dissociation rate of a complex that contained a second peptide that differed in sequence only at the position of the coefficient. Eight other coefficients were allowed to be variable (D at P1; R and W at P3; W and Y at P5; W and Y at P6; and W at P7) because they correspond to aa that are chemically similar to aa that are expected to be important for binding based on the singly substituted peptide studies described above. Three coefficients at P4 (corresponding to Ile, Leu, and Phe) were allowed to be variable because peptides containing these aa formed complexes that were less stable than could otherwise be accounted for. All other coefficients were fixed at 1.0. A fortran program was written based on the algorithm of Davidon (18) to solve for all 82 variables simultaneously. A second program was written that further optimized each coefficient separately starting with the output from the first program. In addition, this program determined the maximum tolerable value for a coefficient that was present only in the set of nonbinding peptides. The coefficients (see Table V) were obtained using these two programs starting with the assumption that each coefficient (and Constant) had a value of 1.0. Almost all nonapeptides for which we have collected data were used in the calculations, and are listed (see Tables II–IV). There were two classes of exceptions. Some peptides contained such poor dominant anchor residues at P2 and P9 that including them would have no impact on the calculations. The second class of exceptions (see Table VI) were excluded because they were not consistent with the bulk of the data. Three of these peptides (see Table VIA) probably violate the requirement of independent binding of side-chains (see below). The remaining 12 peptides (see Table VIB) contained poor dominant anchor residues at P2 and P9 or poor auxiliary anchor residues at P3, and often formed complexes in relatively low yield, or highly variable yield. It is possible that the active species in some of the peptides (see Table VIB) are contaminating peptides. We are currently investigating the basis for the unusual behavior of these peptides.

Comparison of experimental to theoretical

To test how well the coefficients fit the experimental data, theoretical β_2m dissociation rates were calculated from the table of coefficients (see Table V) and the overall normalization constant. In practice, this was done using a program written using Dbase III software (Ashton-Tate,

Torrance, CA). In this program, the inputs are the table of coefficients to be used, a table containing peptide sequences (in single aa code) and experimental binding data. The program calculates the theoretical dissociation rate and the ratio of the experimental to theoretical.

Ranking of potential HLA-A2 binding peptides

Software was written in the Dbase III programming language that generates a table containing the sequence of every possible contiguous nine aa peptide starting from a protein's primary sequence. The peptides can then be indexed according to the theoretical β_2m dissociation rate, calculated using the coefficients (see Table V).

Results

β_2m dissociation rate data for pairs of peptides that differ at single aa

It has been found that the β_2m dissociation rate from HLA-A2 complexes containing peptides with a Leu at P2 and a Val at P9 varies over at least four orders of magnitude, depending on the sequence of the rest of the peptide (17). Nonetheless, the Leu at P2 in either peptide GLGGGGGGV ($t_{1/2}$ < 1 min) and in peptide LLFGYPVYV ($t_{1/2}$ 4000 min) might stabilize the corresponding HLA-A2 complex to the same degree. One way to test this idea is to compare the β_2m dissociation rates of pairs of peptides that differ in sequence by a single aa substitution. Table I contains data of this kind, listed according to the position within the peptide, and then alphabetically according to the single letter aa code of the aa to be compared (first column). For example, at the top of Table I, there are five pairs of peptides that differ only by substitution of Lys for Gly at P1. The ratio of β_2m dissociation rates obtained for HLA-A2 complexes containing each pair of peptides is shown in Table I, second column. It can be seen that, in this instance, the ratio of the rate constants falls within a rather narrow range, from 2.5 to 6.1. Similarly, peptides that contain Leu at P2 bind between 100 and 200 times better than the corresponding Ala peptide, whereas Ile peptides bind about 15- to 25-fold worse than the corresponding Leu peptides. In most cases, of the 15 combinations of aa that are compared in Table I, the range in the ratio observed is considerably less than an order of magnitude. The variability in these ratios may be due to experimental error (especially in cases where one or both dissociation rates are <10 min or more than 2000 min) or may be dependent on the other amino acids present in the same peptide (see *Discussion*).

Table I. Comparison of β2m dissociation rates of HLA-A2 complexes containing peptides differing by one amino acid at given position

aa1/aa2[a]	Ratio[b]	Peptide 1		Peptide 2	
		Sequence	$t_{1/2}$[c]	Sequence	$t_{1/2}$[c]
K1/G1	4.6	KALGFVFTL	410	GALGFVFTL	89
K1/G1	2.5	KILGFVFTL	2500	GILGFVFTL	1000
K1/G1	4.8	KILGKVFTL	2100	GILGKVFTL	440
K1/G1	4.3	KLFGGGGGV	470	GLFGGGGGV	110
K1/G1	6.1	KLFGGVGGV	730	GLFGGVGGV	120
L2/A2	>24	GLFGGVGGV	120	GAFGGVGGV	<5
L2/A2	170	GLLGFVFTL	15000	GALGFVFTL	89
L2/I2	>22	GLFGGGGGV	110	GIFGGGGGV	<5
L2/I2	>24	GLFGGVGGV	120	GIFGGVGGV	<5
L2/I2	15	GLLGFVFTL	15000	GILGFVFTL	1000
L2/Q2	11	GLFGGVGGV	120	GQFGGVGGV	11
L2/Q2	37	GLLGFVFTL	15000	GQLGFVFTL	410
L2/Q2	3.4	LLFGYPVYV	6400	LQFGYPVYV	1900
L2/M2	5	GLFGGGGGV	110	GMFGGGGGV	22
L2/M2	1.4	GLFGGVGGV	120	GMFGGVGGV	84
L2/M2	.88	LLFGYPVYV	6400	LMFGYPVYV	7300
A3/E3	10	GIAGFVFTL	240	GIEGFVFTL	24
A3/E3	43	ILASLFAAV	348	ILESLFAAV	8.1
F3/G3	>100	GLFGGGGGV	110	GLGGGGGGV	N/A
F3/G3	>24	GLFGGVGGV	120	GLGGGVGGV	<5
F3/G3	>400	GLFGGGFGV	2000	GLGGGGFGV	<5
F3/G3	>36	GLFGGFGGV	180	GLGGGFGGV	<5
F3/L3	>22	GLFGGGVGV	110	GLLGGGVGV	<5
F3/L3	9.2	GLFGGGVGV	830	GLLGGGVGV	90
G4/K4	4.0	GILGFVFTL	1000	GILKFVFTL	250
G4/K4	1.1	GLFGGGGGV	110	GLFKGGGGV	104
F5/K5	2.3	GILGFVFTL	1000	GILGKVFTL	440
F5/K5	1.2	KILGFVFTL	2500	KILGKVFTL	2100
V6/G6	1.1	GLFGGVGGV	120	GLFGGGGGV	110
V6/G6	3.8	GMFGGVGGV	84	GMFGGGGGV	22
V6/G6	1.6	KLFGGVGGV	730	KLFGGGGGV	470
F7/A7	10.3	GILGFVFTL	1000	GILGFVATL	97
F7/A7	2.6	GLFGGGFGV	2000	GLFGGGAGV	770
F7/E7	15.0	GILGFVFTL	1000	GILGFVETL	65
F7/E7	3.8	GLFGGGFGV	2000	GLFGGGEGV	530
V7/G7	7.5	GLFGGGVGV	830	GLFGGGGGV	110
V7/G7	>18	GLLGGGVGV	90	GLLGGGGGV	<5
L9/A9	1.8	GILGFVFTL	1000	GILGFVFTA	550
L9/A9	>4.8	GLFGGGGGL	24	GLFGGGGGA	<5

[a] aa, using single letter code, and peptide position to be compared.
[b] $t_{1/2}$ of the first peptide divided by $t_{1/2}$ *of the second peptide.*
[c] Half-life of β_2m dissociation for HLA-A2 complexes containing the peptide, in min at 37°C.

Prediction of β_2m dissociation rates using coefficients specific for each combination of aa and peptide position

When a given peptide binds to a class I molecule, the stability of the complex can theoretically be divided into coefficients that represent the contribution of each aa within the peptide to the overall stability. To the first approximation, each of these coefficients could be independent of the sequence of the rest of the peptide; that is, there could be IBS. The ratio in Table I, second column, can be thought of as the ratio of the corresponding coefficients (see Table V). To test the IBS idea, the coefficients must be experimentally determined for a reasonably large set of peptides. For a 9 aa peptide, there would be 180 (9 residues × 20 aa) possible coefficients. For our initial cal-

culations, we chose to limit the number of variables by solving for only those coefficients that are most important for binding to HLA-A2 (82 coefficients in all), based on the peptides that we have studied. All other coefficients were assigned a neutral value of 1.0.

We calculated these coefficients from data on the stability of HLA-A2 complexes containing individual peptides, and also took account of which peptides did not bind to HLA-A2. Peptides that form HLA-A2 complexes can be distinguished from nonbinding peptides by use of a GF assay in which the ability of each peptide to promote incorporation of ^{125}I-β_2m into HLA-A2 complexes is assessed. Average percentages of peptide-dependent β_2m incorporation are listed in column 2 of Tables II, III, IV, and VI. The peptides that formed HLA-A2 complexes could be further subdivided according to how stable the complexes were once they were formed. Those peptides that formed complexes that had a half-life of dissociation of >5 min at 37°C (see column 3) are listed in Table II, whereas those peptides that formed less stable complexes are listed in Table III. Peptides that caused the incorporation of less than 10% of the labeled β_2m into HLA-A2 complex when assayed at a concentration of 1 mM were considered to be *nonbinders* for HLA-A2, and are listed in Table IV. In the calculations, each peptide in Table II corresponded to one independent equation; in which the product of the appropriate coefficients and an overall normalization constant was set equal to the experimentally measured half-life (see *Materials and Methods*). In contrast, the peptides in Table III and Table IV corresponded to an inequality; in which the product of the appropriate coefficients and the overall normalization constant was set equal to less than a half-life of 5 min. For the purpose of discussion, each category of peptide was further divided by sequence into three categories; those based on a poly-Gly or poly-Ala backbone (Tables IIA, IIIA, and IVA), those related to the M1 peptide, which is an optimal HLA-A2 restricted antigenic influenza A matrix peptide (Table IIB and IVB), and other peptides (Table IIC, IIIB, and IVC), including HLA-A2-restricted antigenic peptides and essentially random viral peptides that we had synthesized.

When the equations and inequalities corresponding to each of the peptides listed in Tables II, III, and IV were simultaneously solved, the coefficients listed in Table V were obtained. These coefficients were then used to calculate the theoretical half-life of dissociation of β_2m listed in the column labeled "theo" in Tables II, III, and IV. It can be seen from the ratio listed in the fifth column of Table II that, in every case where accurate experimental half-lives are obtainable, the theoretical binding stabilities differ from the actual binding stabilities by less than a factor of 5.0, and the average ratio is a factor of 1.6. The overall fit of the data is shown graphically in Figure 1*A*, where the theoretical half-life of β_2m dissociation is plotted vs the experimental half-life. Considering that these rate constants vary over at least four orders of magnitude, the fit is impressive. A ratio of coefficients similar to that listed in Table I, second column, can be calculated from the coefficients in Table V. For example, the ratio of coefficients from Table V for K1/G1 is 3.465/0.578 = 6.0, compared to a range of between 2.5 and 6.1 as shown in Table I, second column. This verifies that the coefficients shown in Table V faithfully reflect the contribution of each aa of a nonamer peptide for binding to HLA-A2. The fact that the majority of the half-lives is predicted well (Fig. 1*A*) supports the premise that side-chain/side-chain interactions are in the majority of cases of minimal importance in peptide binding.

Two features of the coefficients listed in Table V are of particular relevance. First, the most important coefficients in Table V are those that are significantly different from 1.0. Second, the higher the frequency of the coefficient among the equations, the more accurately known the value of the coefficient. This is because the value of a coefficient has a greater impact on the overall error if the coefficient is present in a large number of equations, especially if the peptides that correspond to those equations form stable HLA-A2 complexes. The frequency of each aa/peptide position combination in peptides that form stable HLA-A2 complexes (Table II) and in peptides that do not form stable complexes (Tables III and IV) is listed in parentheses in Table V. It can be seen that the coefficient for L2 is both important and accurately known, because it has a high numerical value (103.183), and it appears in 33 different equations (corresponding to 33 peptides that form stable HLA-A2 complexes), and in 39 additional inequalities (corresponding to 39 different peptides that either form unstable HLA-A2 complexes, or do not bind at all). In contrast, the coefficient for K2 is more tentative, because only one peptide containing a Lys at P2 was tested that formed a stable HLA-A2 complex[3]. To get the most accurate values for the coefficients, we included in the calculations equations corresponding to as large a number of peptides as possible, because each additional peptide adds an additional constraint to the values of nine different coefficients. However, there were certain peptides that were excluded from the set (listed in Table VI) because their binding properties appeared to be inconsistent with the bulk of the peptides. In particular, three peptides stood out (Table VIA) that bound reasonably well to HLA-A2 and formed complexes in high yield. These three peptides, we believe, violate the assumption of independent binding of side-chains (see below). The remainder of the peptides were excluded because the data seemed in some way to be "dubious" (Table VIB). Some of these peptides promoted the incorporation of a rather small percentage of ^{125}I-β_2m into complexes. One possible explanation for this could be that a contaminant in the peptide preparation is the active

[3] Analysis of the stability of complexes containing five additional peptides that contain Lys at P2 indicates that the coefficient for Lys at P2 as listed in Table V is too high, and should be close to 1.0.

Table II. A. Poly-Gly and poly-Ala nonapeptides that form stable HLA-A2 complexes

Sequence[a]	GF[b]	Expt. $t_{1/2}$[c]	Theoretical		Predicted	
			$t_{1/2}$[d]	Ratio[e]	$t_{1/2}$[f]	Ratio[g]
ALFAAAAAV	70	570	870	1.5	1100	2.0
GIFGGVGGV	70	8	11	1.4	12	1.6
GLDKGGGGV	70	6	3	2.4	0.6	10
GLFGGFGGV	80	180	190	1.0	180	1.0
GLFGGGAGV	80	770	430	1.8	270	2.9
GLFGGGEGV	90	530	350	1.5	190	2.9
GLFGGGFGV	90	2000	2700	1.3	3300	1.6
GLFGGGGGL	70	24	22	1.1	21	1.2
GLFGGGGGV	70	110	45	2.5	39	2.8
GLFGGGVGV	90	830	480	1.7	230	3.6
GLFGGVGGV	80	120	110	1.1	110	1.2
GLFGGVGKV	80	96	110	1.1	110	1.2
GLFKGVGGV	80	100	110	1.1	110	1.1
GLGGGGFGV	70	8	21	2.8	120	16
GLLGGGVGV	90	90	160	1.7	300	3.4
GLYGGGGGV	60	140	30	4.5	4.1	33
GMFGGGGGV	90	22	25	1.1	27	1.2
GMFGGVGGV	70	84	61	1.4	50	1.7
GQFGGVGGV	70	8	11	1.3	13	1.6
GVFGGVGGV	60	6	6	1.0	1.1	6.4
KLFGGGGGV	90	470	270	1.7	210	2.2
KLFGGVGGV	80	730	660	1.1	630	1.2
B. M1-related nonapeptides that form stable HLA-A2 complexes						
AILGFVFTL	80	1800	1300	1.4	1100	1.6
GAIGFVFTL	80	38	38	1.0	20	1.9
GALGFVFTL	50	89	76	1.2	59	1.5
GELGFVFTL	60	280	280	1.0	180	1.5
GIAGFVFTL	50	240	210	1.1	220	1.1
GIEGFVFTL	60	21	11	1.8	4.3	4.9
GILAFVFTL	40	610	770	1.3	760	1.2
GILGAVFTL	50	220	130	1.7	78	2.8
GILGEVFTL	80	220	96	2.3	14	15
GILFGAFTL	60	220	300	1.4	360	1.6
GILGFEFTL	80	730	960	1.3	2100	2.9
GILGFKFTL	50	71	71	1.0	300	4.2
GILGFVATL	50	97	120	1.2	130	1.3
GILGFVETL	70	65	99	1.5	180	2.7
GILGFVFAL	40	1000	770	1.3	740	1.4
GILGFVFEL	90	870	770	1.1	750	1.2
GILGFVFKL	90	1200	720	1.5	730	1.6
GILGFVFTA	70	560	330	1.7	30	19
GILGFVFTL	50	1000	770	1.3	760	1.4
GILGFVFVL	90	210	230	1.1	310	1.5
GILGFVKTL	50	68	73	1.1	93	1.4
GILGKVFTL	70	440	270	1.7	170	2.6
GILKFVFTL	80	250	770	3.1	820	3.3
GILPFVFTL	90	1100	770	1.5	750	1.5
GIVGFVFTL	80	180	450	2.5	940	5.2
GKLGFVFTL	30	1500	1600	1.0	75	21
GLLGFVFTL	80	15000	7800	2.0	6000	2.6
GQLGFVFTL	90	410	760	1.9	1500	3.6
KALGFVFTL	90	380	450	1.2	540	1.4
KILGFVFTL	50	2700	4600	1.7	5600	2.1
KILGKVFTL	90	2100	1600	1.3	1200	1.8
C. Other nonapeptides that form stable HLA-A2 complexes						
AILLGVFML	20	54	110	2.1	440	8.2
AIYKRWIIL	60	7	7	1.0	340	48
ALFFFDIDL	30	67	67	1.0	41000	610
ATVELLSFL	70	6	5	1.3	3.1	2.0
CLFGYPVYV	90	4600	4600	1.0	7700	1.7

[a] Sequence in single-letter aa code.
[b] Average % of β_2m incorporation as assessed by gel filtration.
[c] Experimentally measured half-life of β_2m dissociation in min at 37°C.
[d] Theoretical half-life of β_2m dissociation, calculated using coefficients in Table V.
[e] Factor by which the theoretical half-life differs from the measured half-life: (column 4 ÷ column 3, or column 3 ÷ column 4, whichever is >1).
[f] Predicted half-life of β_2m dissociation using coefficients that were calculated from all of the equations except the equation corresponding to the peptide that is being predicted.
[g] Factor by which the predicted half-life differs from the measured half-life: (column 6 ÷ column 3, or column 3 ÷ column 6, whichever is >1).

Table II—Continued

Sequence[a]	GF[b]	Expt. $t_{1/2}$[c]	Theoretical $t_{1/2}$[d]	Theoretical Ratio[e]	Predicted $t_{1/2}$[f]	Predicted Ratio[g]
C. Other nonapeptides that form stable HLA-A2 complexes (*Continued*)						
FIFPNYTIV	90	200	300	1.5	670	3.4
IISLWDQSL	60	6	6	1.1	9.8	1.7
ILASLFAAV	70	350	330	1.0	340	1.1
ILESLFAAV	70	10	18	1.8	45	4.7
KLGEFFNQM	90	350	190	1.8	44	7.9
KLGEFYNQM	80	220	150	1.5	57	3.9
KMFGYPVYV	90	4400	15000	3.4	58000	13
LLFGYPVYV	70	6400	7800	1.2	9000	1.4
LLWKGEGAV	80	360	270	1.3	120	3.1
LMFGYPVYV	90	7300	4400	1.7	3100	2.4
LNFGYPVYV	40	41	41	1.0	350	8.6
LQFGYPVYV	90	1800	750	2.5	270	6.9
NIVAHTFKV	80	140	100	1.4	75	1.9
NLVPMVATV	90	480	430	1.1	360	1.3
QMLLAIARL	80	110	49	2.3	13	8.5
QMWQARLTV	90	420	560	1.3	1400	3.3
RLLQTGIHV	90	300	330	1.0	280	1.1
RLVNGSLAL	70	81	64	1.3	36	2.3
SLYNTVATL	80	370	720	1.9	2100	5.6
TLNAWVKVV	70	100	94	1.1	70	1.4
WLYRETCNL	80	91	210	2.3	1600	18
YLFKRMIDL	90	570	420	1.4	390	1.5

species, as has been found in other assay systems (19). In most cases, dissociation rates for the peptides listed in Table VIB were difficult to calculate because the majority of the counts incorporated into HLA-A2 complex dissociated rapidly, although a small percentage of the complex dissociated with the half-life listed. For ALFAAAAAY and GQLGFVFTK no internally consistent half-life could be obtained. In addition, all of these peptides have anchor residues at P2, P3, or P9 (marked in bold in Table VIB), that are infrequent or absent among peptides that form stable HLA-A2 complexes. For all of these reasons, we believe it would be wisest to exclude these peptides from the calculations for the time being, until more peptides are synthesized and tested that could help address these problems.

Explanation of the values obtained for the coefficients

The coefficients in Table V corroborate the data obtained previously from endogenous peptide sequence analyses (5, 8) that the most important anchor positions are at P2 and P9. In addition to Leu and Met at P2, Ile and Gln are also relatively well tolerated. Although Gln has not been previously reported to be an anchor residue at P2 for wild-type HLA-A2, it was recently found to be present at P2 in pooled endogenous peptides isolated from mutant HLA-A*0205 molecules (20), which differ from A*0201 molecules by a single substitution (F9Y) in the B pocket. Our data suggest that the most abundant anchor residues at P2 for this mutant are different from wild type HLA-A2 in a quantitative sense only. At P1, negatively charged residues are unfavorable, whereas Lys is favorable. This can most easily be explained by an ionic interaction with E63, which is known to be located near the N-terminus of the peptide-binding site (4). At P3, aromatic residues are favorable, and charged residues are most often unfavorable. A few exceptional peptides, notably ILDKKVEKV and ILKEPVHGV, can form stable HLA-A2 complexes despite the charged residue at P3, presumably by means of overriding favorable interactions with other peptide residues (a violation of the IBS condition). Most residues are equally well tolerated at P4; however, our data tentatively indicate that large hydrophobic residues like Phe are unfavorable. At P5–P7, aromatic residues seem to be favored, as at P3; however, KLFGFVFTV, which contains Phe at P3, P5, and P7, binds much less well than would be predicted (2,000 min vs 300,000 min predicted) if each of these positions contributed independently. Most likely, this is due to the limited space that is available within the peptide-binding groove to accommodate bulky side-chains (this would be a second violation of the IBS principle). At P8, Val is significantly less favorable than Ala, Glu, Lys, or Thr, at least in the context of the matrix peptide sequence (GILGFVFTL). This may indicate that the hydrophobic isopropyl group of Val cannot be accommodated as easily as hydrophilic, or smaller side-chains. At P9, Val and Leu are better than Met and Ile, and all other residues examined appear to be very much worse. The importance of the P9 position is exemplified by the data collected using peptides that belong to the paradigm GLFGGGFGX, because GLFGGGFGF, GLFGGGFGN, and GLFGGGFGS form complexes that are at least 1000-fold less stable than GLFGGGFGV. Moreover, most peptides that contain either Lys or Tyr at P9 do not bind appreciably, despite

Table III. A. Poly-Gly and poly-Ala nonapeptides that form unstable HLA-A2 complexes

Sequence[a]	GF[b]	$t_{1/2}$[c]	Theo[d]	Ratio[e]
GAFGGVGGV	20		1.1	
GAFGGVGGY	30		0.002	
GEFGGVGGV	20		4.0	
GGFGGVGGV	10		0.10	
GIFGGGGGV	40	3	4.4	1.3
GIGGFGGGL	20		0.12	
GIGGGGGGL	20		0.016	
GLDGGGGGV	60	4	2.9	1.5
GLDGKGGGV	10		7.4	
GLDKKGGGV	30	1	7.4	7.4
GLFGGGFGF	70	5	4.9	1.1
GLFGGGFGG	50	1	4.9	4.9
GLFGGGFGN	80	3	4.9	1.5
GLFGGGFGS	60	1	4.9	4.9
GLFGGGGGA	50	1	9.2	6.6
GLFGGGGGI	70	4	4.9	1.1
GLFGGGGGM	60	4	14	3.1
GLFGGGGGT	50		0.082	
GLFGGGGGY	10		0.083	
GLGFGGGGV	40	2	0.009	250.0
GLGGFGGGV	60	5	2.6	1.8
GLGGGFGGV	60	3	1.4	2.3
GLGGGGGFV	60	3	0.34	7.5
GLGGGGGGY	10		0.001	
GLGGGVGGV	40	1	0.084	1.1
GLLGGGGGV	50	3	15	5.2
GLPGGGGGV	40		5.0	
GNFGGVGGV	10		0.58	
GSFGGVGGV	20		5.0	
GTFGGVGGV	40	1	6.4	4.3
B. Other nonapeptides that form unstable HLA-A2 complexes				
AGNSAYEYV	10		0.21	
GLFPGQFAY	10		4.8	
HILLGVFML	10		5.0	
ILESLFRAV	20	2	5.0	2.7
KKKYKLKHI	10		0.18	
MLASIDLKY	20		0.14	
MLERELVRK	10		0.011	

[a] Sequence, in single-letter aa code.
[b] Average % of β_2m incorporation as assessed by gel filtration.
[c] Experimentally measured half-life of β_2m dissociation in min at 37°C. If no number is present, the half-life was difficult to measure, but is probably less than 5 min.
[d] Theoretical half-life of β_2m dissociation, calculated using coefficients in Table V.
[e] Factor by which the theoretical half-life differs from the measured half-life.

Table IV. A. Poly-Gly and poly-Ala nonapeptides that do not bind to HLA-A2

Sequence[a]	GF[b]	Theo[c]
ALAAAAAAK	1	0.24
GDFGGVGGV	4	5.0
GFFGGVGGV	5	5.0
GHFGGVGGV	8	5.0
GIFGGGGGA	9	0.90
GIGGGFGGL	3	0.068
GIGGGGFGL	2	1.0
GLFGGGGGF	6	0.08
GLGGGGGGL	4	0.17
GLGGGGGGV	5	0.34
GPFGGVGGV	5	5.0
GRLGGGGGI	5	0.036
GYFGGVGGV	6	5.0
B. M1-related nonapeptides that do not bind to HLA-A2		
EILGFVFTK	0	5.0
GILGFVFTE	2	4.9
GILGFVFTK	4	5.0
C. Other nonapeptides that do not bind to HLA-A2		
DIYRIFAEL	4	5.0
EIKDTKEAL	3	0.01
EIYKRWIIL	7	4.0
ELDAPNSHY	1	0.059
ELKSKYWAI	1	5.1
ELKVKNLEL	2	1.1
ELRSLYNTV	2	5.0
ELRSRYWAI	3	3.3
ERYLKDQQL	4	4.9
GEIYKRWII	5	4.0
GLPVGGNEK	5	0.15
GMQWNSTAF	4	0.044
ILKQKIADL	8	1.9
ILRGSVAHK	7	0.020
KIFIAGNSA	1	5.0
KLYEKVYTY	3	5.0
LGFVFTLTV	5	5.0
LLSFLPSDF	5	0.004
PLNPFVSHK	1	3.7
RYWAIRTRS	2	0.082
TPQDLNTML	3	1.7

[a] Sequence, in single letter aa code.
[b] Average % of β_2m incorporation, as assessed by gel filtration.
[c] Theoretical half-life of β_2m dissociation, calculated using coefficients in Table V.

otherwise very favorable residues (e.g., GILGFVFTK, KLYEKVYTY; see Table IVB and IVC).

Application of the binding coefficients to ranking of known antigenic and endogenous peptides

It would be interesting to know if the known endogenously synthesized self and antigenic peptides are among the best HLA-A2 binding peptides. Theoretically, large numbers of peptides may be more capable of binding to HLA-A2, but might never be generated in vivo. To determine whether this is likely, the coefficients in Table V were used to rank all of the potential nonamers from each of the proteins for which a known antigenic or endogenous peptide has been identified. The parameters that describe the ranking of each peptide are shown in Table VII. Column 3 shows the number of overlapping nonamers that could be generated from each protein. Column 4 shows the theoretical half-life of β_2m dissociation for the most stable nonamer. The next two columns list the rank of the peptide using the experimentally measured half-life of dissociation, followed by the measured half-life. Finally, the last two columns list the rank of peptide when the peptide's theoretical half-life of dissociation is used, followed by the theoretical half-life. (Note that our current algorithm is capable of ranking nonamers only, although some longer peptides could form comparably stable complexes.) The first peptide, the influenza matrix peptide GILGFVFTL, was previously found to be a major target of all HLA-A2-restricted, influenza-specific CTL, both in humans (21) and in HLA-A2 transgenic mice (22). It ranks first among all possible nonamers from the matrix protein

Table V. Coefficients used to calculate theoretical rate constants

aa[a]	Coeff[b]	Freq[c]	aa	Coeff	Freq	aa	Coeff	Freq
A1	1.000	(6, 2)	A4	1.000	(4, 3)	A7	1.000	(8,4)
C1	0.597	(1, 0)	C4	1.000	(0, 0)	C7	1.000	(1, 0)
D1	0.041	(0, 1)	D4	1.000	(0, 2)	D7	1.000	(0, 0)
E1	0.578	(0, 9)	E4	1.000	(3, 1)	E7	0.820	(2, 2)
F1	1.000	(1, 0)	F4	0.027	(1, 2)	F7	6.383	(32, 10)
G1	0.578	(46, 48)	G4	1.000	(53, 44)	G7	0.105	(16, 37)
H1	0.044	(0, 1)	H4	1.000	(0, 0)	H7	1.000	(0, 0)
I1	1.000	(3, 3)	I4	0.078	(0, 1)	I7	1.000	(4, 1)
K1	3.465	(8, 3)	K4	1.000	(6, 2)	K7	0.603	(2, 1)
L1	1.000	(5, 2)	L4	0.646	(3, 2)	L7	1.000	(2, 3)
M1	1.000	(0, 2)	M4	1.000	(0, 0)	M7	1.000	(0, 0)
N1	1.000	(2, 0)	N4	1.000	(2, 0)	N7	1.000	(2, 3)
P1	1.000	(0, 1)	P4	1.000	(3, 2)	P7	1.000	(0, 0)
Q1	1.000	(2, 0)	Q4	1.000	(2, 1)	Q7	1.000	(1, 1)
R1	1.000	(2, 1)	R4	1.000	(1, 2)	R7	0.277	(0, 1)
S1	1.000	(1, 0)	S4	1.000	(2, 6)	S7	1.000	(1, 3)
T1	1.000	(1, 1)	T4	1.000	(0, 0)	T7	1.000	(1, 3)
V1	1.000	(0, 0)	V4	1.000	(0, 3)	V7	1.120	(8, 1)
W1	1.000	(1, 0)	W4	1.000	(0, 1)	W7	5.951	(0, 3)
Y1	1.000	(1, 0)	Y4	1.000	(0, 2)	Y7	1.000	(0, 1)
A2	1.000	(3, 2)	A5	1.000	(4, 3)	A8	1.000	(6, 7)
C2	0.500	(0, 0)	C5	1.000	(0, 0)	C8	1.000	(0, 0)
D2	0.500	(0, 1)	D5	1.000	(0, 0)	D8	1.000	(2, 2)
E2	2.840	(1, 2)	E5	0.756	(2, 1)	E8	1.000	(1, 3)
F2	0.500	(0, 1)	F5	6.044	(30, 7)	F8	1.000	(1, 1)
G2	0.500	(0, 3)	G5	0.804	(24, 41)	G8	1.000	(20, 41)
H2	0.500	(0, 1)	H5	1.000	(1, 0)	H8	1.000	(1, 4)
I2	10.151	(30, 14)	I5	1.000	(0, 3)	I8	1.000	(2, 2)
K2	20.524[3]	(1, 1)	K5	2.085	(2, 9)	K8	1.000	(3, 1)
L2	103.183	(33, 39)	L5	1.000	(3, 4)	L8	1.000	(0, 0)
M2	57.920	(6, 1)	M5	1.000	(1, 0)	M8	1.000	(1, 2)
N2	0.542	(1, 1)	N5	1.000	(1, 1)	N8	1.000	(1, 0)
P2	0.500	(0, 2)	P5	1.000	(0, 1)	P8	1.000	(0, 0)
Q2	10.006	(3, 0)	Q5	1.000	(0, 0)	Q8	1.000	(2, 1)
R2	0.500	(0, 2)	R5	1.000	(2, 2)	R8	1.000	(1, 2)
S2	0.500	(0, 1)	S5	1.000	(0, 1)	S8	1.000	(1, 1)
T2	6.080	(1, 1)	T5	1.000	(2, 1)	T8	1.000	(30, 6)
V2	5.919	(1, 0)	V5	1.000	(0, 0)	V8	0.293	(2, 0)
W2	0.500	(0, 0)	W5	2.680	(2, 0)	W8	1.000	(0, 0)
Y2	0.500	(0, 2)	Y5	8.002	(6, 0)	Y8	1.000	(6, 1)
A3	1.000	(2, 2)	A6	1.000	(2, 1)	A9	1.000	(1, 3)
C3	1.000	(0, 0)	C6	1.000	(0, 0)	C9	0.010	(0, 0)
D3	0.726	(1, 4)	D6	1.000	(2, 2)	D9	0.010	(0, 0)
E3	0.054	(2, 2)	E6	3.246	(2, 0)	E9	0.015	(0, 1)
F3	11.383	(27, 27)	F6	4.369	(4, 4)	F9	0.009	(0, 4)
G3	0.088	(3, 12)	G6	1.060	(13, 29)	G9	0.009	(0, 1)
H3	1.000	(0, 0)	H6	1.000	(0, 0)	H9	0.010	(0, 0)
I3	1.849	(1, 1)	I6	1.000	(1, 1)	I9	0.534	(0, 6)
K3	0.024	(0, 5)	K6	0.239	(1, 1)	K9	0.015	(0, 7)
L3	3.685	(31, 6)	L6	1.000	(1, 2)	L9	2.357	(41, 13)
M3	1.000	(0, 0)	M6	1.000	(1, 0)	M9	1.501	(2, 1)
N3	1.000	(1, 2)	N6	1.000	(0, 3)	N9	0.009	(0, 1)
P3	1.261	(0, 2)	P6	1.000	(6, 1)	P9	0.010	(0, 0)
Q3	1.000	(0, 2)	Q6	1.000	(0, 1)	Q9	0.010	(0, 0)
R3	0.033	(0, 3)	R6	1.000	(1, 2)	R9	0.010	(0, 0)
S3	1.000	(1, 1)	S6	1.000	(1, 1)	S9	0.009	(0, 2)
T3	1.000	(0, 0)	T6	1.000	(2, 1)	T9	0.009	(0, 1)
V3	2.173	(5, 0)	V6	2.588	(40, 20)	V9	4.884	(36, 27)
W3	12.978	(2, 1)	W6	0.251	(1, 1)	W9	0.010	(0, 0)
Y3	7.613	(4, 4)	Y6	3.470	(2, 4)	Y9	0.009	(0, 7)

[a] aa using single letter code, followed by the position within the peptide.

[b] Coefficient, calculated by solving simultaneously equations corresponding to each of the peptides in Tables II, III, and IV. Coefficients whose value equal exactly 1.000 were constrained to equal 1.0. No coefficient is known to better than two decimal places; many coefficients may be off by greater than a factor of 2.0. The value in this table is representative of the raw output from the Fortran program. At P2, coefficients were assigned a value of 0.500 if no peptides were studied that formed stable complexes with this aa/peptide position combination. At P9, coefficients were assigned a value of 0.010 if no peptides were studied that contained this aa/peptide position combination. Note that all undetermined coefficients in Table V have been assigned the value of 1.0, which corresponds to the coefficient for Ala at that same position. In making predictions of the stability of HLA-A2 complexes containing unknown peptides, one could substitute a coefficient with the corresponding coefficient of a chemically more similar aa. The overall normalization coefficient = 0.151.

[c] First number: number of peptides that contain the aa at the position in question in Table II. Second number: number of peptides that contain the aa at the position in question in Tables III and IV.

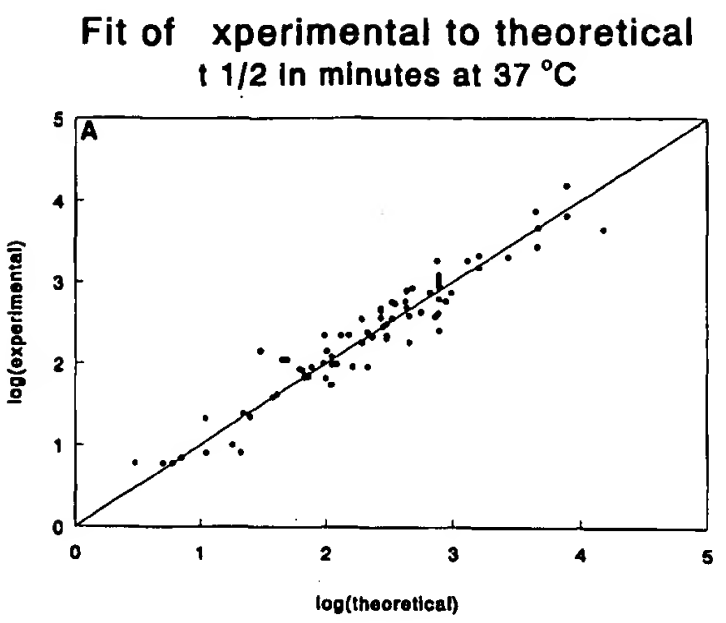

FIGURE 1. *A*, Comparison of theoretical half-life of β_2m dissociation to the experimentally measured half-life. The data from the third column of Table II were plotted against the fourth column. The *line* indicates the position of a perfect fit. *B*, Comparison of the predicted half-life of β_2m dissociation to the experimentally measured half-life. The data from the third column of Table II were plotted against the sixth column. The *line* indicates the position of a perfect fit.

(Table VIIA) and third among all possible nonamers encoded by the influenza genome (data not shown). The HTLV-1 derived, HLA-A2-restricted peptide LLFGYPVYV (23) also ranks first from its source protein. The HIV polymerase-derived, HLA-A2-restricted, antigenic peptide ILKEPVHGV (11) "theoretically" ranks 45th of the 1007 possible nonamers in the pol protein, which would place it only in the top 5%. However, in the case of ILKEPVHGV, the experimental rank is much higher than the theoretical rank, because ILKEPVHGV binds much better than expected based on the coefficients in Table V. Notably, none of the other seven higher-ranking HIV polymerase-encoded peptides are predicted to bind much more than twofold better (data not shown). The remaining three antigenic peptides, KLGEFYNQMM (24), FIAGNSAYEYV (23), and FLPSDFFPSV (25), are longer than nine aa long, which is why no theoretical rank of half-life is listed. When the experimentally measured half-life of these peptides is compared against the theoretical half-lives of all possible nonamers from the source protein, each of these peptides ranks close to the top.

When the endogenous peptides are examined, we see that ILDKKVEKV, like ILKEPVHGV, binds much more tightly than expected using the coefficients in Table V. When its experimentally measured half-life is used for ranking purposes, it ranks at the top of the list. With the exception of LLDVPTAAV, the other endogenous peptides also rank in the top few percent of all possible nonamers from their source protein. Note that our estimates for the ranking of these remaining endogenous peptides are inherently less accurate because we have not measured the half-lives of complexes containing these peptides. The endogenous peptide that ranks the lowest, LLDVPTAAV, was derived from the leader peptide of IP30, and was isolated from a cell line with a mutation in Ag processing, so that presumably only peptides derived from the leader peptide were available for binding to HLA-A2 (26). We conclude that most antigenic peptides and most predominant self peptides are selected from among those peptides that can form the most stable class I complexes.

Table VI. A. Nonapeptides that may violate the side-chain independence rule

Sequence[a]	GF[b]	$t_{1/2}$[c]	Theo[d]	Ratio[e]
KLFGFVFTV	60	2000	300,000	150
ILDKKVEKV	50	2900	250	12
ILKEPVHGV	80	190	4.8	38
B. Peptides that form HLA-A2 complexes that behave irregularly				
ALFAAAAAY	30		2	
GIGFGGGGL	20	200	0	200000
GKFGGVGGV	10	80	22	3.7
GLFGGGGGK	30		0	
EILGFVFTL[f]	10	85	770	9.1
GIKGFVFTL[g]	60	2500	5	500
GQLGFVFTK	70		5	
ILGFVFTLT[h]	50	140	0	
KILGFVFTK	5	210	30	7.2
KKLGFVFTL	30	750	9300	13
KLFEKVYNY	20	9	8	1.2
LRFGYPVYV	20	400	180	2.3

[a] Sequence, in single letter aa code.
[b] Average % of β_2m incorporation, as assessed by gel filtration.
[c] Experimentally measured half-life of β_2m dissociation in min at 37°C.
[d] Theoretical half-life of β_2m dissociation, calculated using coefficients in Table V.
[e] Factor by which the theoretical half-life differs from the measured half-life.
[f] This peptide would have been placed in Table IIB if it were better able to form HLA-A2 complexes. The dissociation rate of complexes containing this peptide is consistent with the rest of the data, but it was not used to calculate the coefficients.
[g] This peptide reproducibly fails to form complexes with the expected pI. Instead, the complex has the same charge as GILGFVFTL complexes.
[h] This peptide is likely to be contaminated with trace amounts of ILGFVFTL, which is known to form complexes with the measured stability (16).

Table VII. Ranking of HLA-A2 antigenic and endogenous peptides using the coefficients[a]

Protein[b]	Sequence[c]	No. 9-mers[d]	Highest $t_{1/2}$[e]	Experimental[f]		Theoretical[g]	
				Rank	$t_{1/2}$	Rank	$t_{1/2}$
A. Antigenic peptides							
Flu matrix (10, 21)	GILGFVFTL	244	800	1st	1000	1st	800
HTLV-1 tax (23)	LLFGYPVYV	350	8000	1st	4000	1st	8000
HIV polymerase (11)	ILKEPVHGV	1007	400	8th	190	45th	10
Influenza nucleoprotein (24)	KLGEFYNQMM	552	600	4th	190		
HCMV gB (23)	FIAGNSAYEYV	889	2000	3rd	1000		
Hepatitis core Ag (25)	FLPSDFFPSV	175	400	1st	1500		
B. Endogenous peptides							
hsp 84 (16)	ILDKKVEKV	715	900	1st	2800	19th	20
ip30 (8)	LLDVPTAAV	295	500			7th	60
tis 21 (8)	TLWVDPYEV	150	1000			1st	1000
helicase (8)	YLLPAIVHI	546	400			8th	30
pp61 (8)	SLLPAIVEL	581	900			6th	200
phosphorylase regulatory A (8)	SLLPAIVEL	581	900			6th	200
phosphorylase regulatory B (8)	SLLPAIVEL	567	900			6th	200

[a] There are other examples of sequences that are known to contain HLA-A2-restricted peptides but, in these other cases, the optimal peptide has not been identified (12–14).

[b] Protein of origin.

[c] Amino acid sequence.

[d] Number of nonamers in the protein.

[e] Theoretical half-life of β_2m dissociation (in min at 37°C) for the peptide that ranked first for this protein.

[f] Rank of peptide determined by comparing the experimentally measured half-life of β_2m dissociation to the theoretical half-life of β_2m dissociation for all the nonamers that could be generated from the same protein. These columns are blank for peptides that have not been tested.

[g] Rank of peptide determined by comparing the theoretical half-life of β_2m dissociation to the theoretical half-life of β_2m dissociation for all the nonamers that could be generated from the same protein. These columns are blank for peptides that are longer than nonamers, because we cannot make an accurate prediction of the theoretical half-life of β_2m dissociation for longer peptides.

Discussion

One of the major reasons to study peptide binding to class I molecules is to be able to determine which peptides are likely to be antigenic, starting from the primary sequence of (for example) a viral protein. In addition, it would be useful to know why certain peptides are antigenic, but most peptides are immunologically silent. The data in Table VII suggests that so far as we can tell, dominant antigenic peptides in HLA-A2-restricted immune responses are among those peptides that bind most tightly to HLA-A2. If this turns out to be generally correct, then it should be possible to develop mathematical algorithms to identify most antigenic peptides using approaches similar to that described herein that are tailored to the peptide-binding properties of each histocompatibility Ag.

The class I MHC protein HLA-A2 has been shown to bind certain peptides, generally 9 aa in length, that preferentially contain Leu or Met at P2 and a Val or Leu at the C-terminus (P9) (5, 8). The residues at these two positions have been termed anchor residues (5) because their relative lack of variability indicates that they serve as primary contact points between the peptide and the class I binding site. However, peptides that contain both Leu at P2 and Val at P9 form complexes whose stability spans at least four orders of magnitude (16), indicating that the aa at other positions can serve as auxiliary anchor residues that are critical for peptide binding. Therefore, to make useful predictions about peptide binding affinity, if possible, the contribution of both the dominant and auxiliary anchor residues must be analyzed on a quantitative basis. The simplest approach is to assume that each amino acid side-chain binds independently of the rest of the peptide (the IBS hypothesis). It seems reasonable to expect that for many peptides, the IBS hypothesis will adequately explain peptide binding, and for other peptides, more complicated explanations will be needed to explain peptide binding. Whenever IBS is true, the binding affinity of any nonamer can be broken down into nine different coefficients, each of which is dependent only on the identity of the aa and the position within the peptide. Therefore, a table containing 180 different coefficients would contain the information necessary to calculate a probable binding affinity for any possible nonamer.

To calculate the coefficients, we measured the stability of a large number of HLA-A2 complexes containing distinct peptides, as assessed by measuring the rate of β_2m dissociation. We also compiled a list of peptides that were unable to make stable complexes with HLA-A2. To solve for the coefficients, the β_2m dissociation data for each peptide was treated as an independent equation, in which the measured half-life of β_2m dissociation was set equal to the product of the nine coefficients (see *Materials and Methods*). In theory, a sufficiently large set of peptide binding data could be used to solve for all of the coefficients simultaneously. In practice, we calculated values for the coefficients that were most important to our current peptide database. Until every aa at every position has been

tested, we cannot exclude the possibility that other coefficients may also contribute significantly to peptide binding to HLA-A2. Despite these approximations, we found that for the vast majority of the peptides that we have tested, the binding data were consistent with the IBS hypothesis. Only for 3 of 83 peptides was it necessary to propose significant side-chain/side-chain interactions to explain the observed peptide-binding properties. We conclude that for most peptides, the stability of the HLA-A2/β_2m/peptide complex is what would be expected if each side-chain of the peptide bound independently to the class I molecule.

Compiling a table of peptide binding coefficients based on individual peptide side-chains has several powerful advantages. First, the coefficients in Table V incorporate all of the peptide-binding data that we have collected so far, with a few exceptions (see Table VI). The table of coefficients can then be used to estimate the binding stability of HLA-A2 complexes containing an untested peptide. As soon as additional binding data become available, the new data can be used to refine the accuracy of the table of coefficients. Second, the table can be used to make a quantitative prediction about which aa in a given peptide are of primary importance for binding to HLA-A2. For example, in the case of the influenza matrix peptide GILGFVFTL, the Phe residues at P5 and P7 are predicted to be almost as important as the Ile at P2. This information could be used to predict which substitutions in an antigenic peptide might allow it to bind more tightly to HLA-A2. In some cases, a peptide that binds very weakly to HLA-A2 might be converted into a useful vaccine candidate by this means. Third, experiments can be designed to test every coefficient in the table by measuring the stability of HLA-A2 complexes containing peptides that differ at the aa in question. Fourth, whenever the binding of a peptide is badly predicted by the table of coefficients, one would predict that significant side-chain/side-chain interactions are taking place or that some side-chain is oriented in a significantly different direction than usual.

The most obvious way to test the validity of the coefficients in Table V would be to predict the half-lives of β_2m dissociation for complexes formed with a new set of peptides, and then to compare the predictions against experimental measurements. We have not explicitly done this, because we have used all new information to improve the values of the coefficients. Instead, to test the power of this methodology to predict which peptides would make the most stable HLA-A2 complexes, the coefficients were recalculated for each of the 80 peptides that bind stably to HLA-A2, using all of the equations used to calculate the coefficients in Table V except for the equation corresponding to the peptide to be tested. The factor by which the "predicted" half-life of β_2m dissociation differs from the measured half-life is listed in Table II, seventh column. It can be seen that although this factor is always greater than the factor obtained when the peptide to be tested is included in the set of equations (Table II, fifth column), the half-lives of 62 of the 80 peptides were still predicted within a factor of five. In most cases, the poorest predictions can be easily explained. For example, ALFFFDIDL (Table IIC) was predicted poorly because it was the only peptide that formed stable HLA-A2 complexes that contained a Phe at P4. When the equation for ALFFFDIDL was deleted, the program calculated the highest value for the coefficient for Phe at P4 that was consistent with the observation that GLGFGGGGV (Table IIIA) and LLSFLPSDF (Table IVC) do not form stable HLA-A2 complexes. It turns out that this causes the value of the coefficient for F4 to increase from 0.027 (Table V) to 16.7, which is an artificially high value. This happens because GLGFGGGGV and LLSFLPSDF have such poor anchor residues at P3 and P9, respectively that the coefficient for F4 could be as high as 16.7, and these peptides would still not be expected to form stable HLA-A2 complexes.

As a further check on the logic behind the calculations, the coefficients were recalculated allowing all of the 180 coefficients to be variables. This allowed the overall error function to decrease from a value of 22.8 to a value of 12.3. The new set of coefficients was very similar to that in Table V (data not shown), especially for the coefficients that apply to a large number of peptides (like L2 and V9). As would be expected considering the number of variables, certain coefficients were poorly defined. For example, the coefficients for both W1 and C7 were present only in the equation corresponding to the data for WLYRETCNL (see Table II), and other coefficients could not be calculated at all because peptides containing the corresponding aa were not available. Nonetheless, with a sufficiently large set of peptides, these difficulties would be overcome, and all of the coefficients could be simultaneously calculated. Thus, it will not always be necessary to make intuitive choices as to which coefficients should be allowed to deviate from a value of 1.0. However, it would be possible to reduce the number of variable coefficients used in our calculations by two distinct means. First, some of the coefficients that were allowed to be variables were calculated to have values near 1.0 (e.g., D3, P3, L4, E5, G5, G6, E7, and V7), and therefore (in retrospect) need not have been variables. Second, in some cases, chemically similar aa were found to have similar coefficients, even though the algorithm used to calculate the coefficients did not take this into account (e.g., F3, Y3, W3 and F5, Y5, W5), and therefore the number of variables could be reduced by constraining several coefficients to have the same value.

The IBS hypothesis is based on the following theoretical considerations. The logarithm of each coefficient can be thought of as being related to a partial free energy of activation for the process of dissociation of the complex. The partial free energies of activation should be additive, assuming the peptide side-chains bind independently to the HLA H chain, and assuming the rate-limiting step for

the dissociation of each complex is the same. The sign of the logarithm of a coefficient can be either negative or positive, depending on whether the aa makes a favorable or unfavorable contribution to the stability of the complex. The equation $E = -RT \ln K$ converts from energy, which is additive, to the coefficients themselves, which are factors that contribute to the value of the rate constant. Instead of measuring free energies, we have measured exclusively the complex stability, as deduced from the half-life of β_2m dissociation. These kinetic measurements may have some advantages over free energy measurements, because there is no contribution from variations in free peptide solvation, and the uncertain status of the HLA H chain/β_2m dimer. However, whenever two peptide side-chains interact with one another, compete for binding to the same pocket, transmit structural changes to other pockets, or alter the structure of the rate-limiting step for dissociation, the IBS condition would be violated.

The IBS hypothesis is based on the approximation that any one aa at a given peptide position would be able to adopt nearly the same limited set of conformations, regardless of the rest of the peptide's sequence. The currently available crystal structure data indicate that the overall structure of the peptide-binding groove is similar for all peptide complexes (4, 27–32), and adjusts only slightly to different peptides (28). In particular, the conformation of the peptide is constrained by the canonical hydrogen bonds between the class I molecule and the peptide termini at both end of the peptide-binding groove (28, 30, 32). In addition, one might expect that for HLA-A2 the anchor residue at P2 would always be buried in the B pocket (4). These constraints would be expected to limit the potential flexibility of the peptide. The energetics of the conformations of each aa, and its interactions with the peptide-binding groove would determine the values of the coefficients, which might also incorporate side-chain solvation effects in the case of exposed aa, and also secondary effects transmitted to other residues by limitations to the conformational flexibility of the peptide backbone. Theoretically, energy minimization calculations based on crystallographically determined coordinates of one peptide/H chain complex should be able to quantitate the energetic consequences of substitutions in the peptide, making the table of coefficients obsolete. At this point in time, however, these calculations are cumbersome and unreliable, and the table of coefficients can provide a first approximation to the binding properties of an unknown peptide.

Because it appears that longer peptides can loop out in the middle in order to maintain favorable contacts at both termini (28, 31), it would be possible to extend the IBS idea to account for the binding properties of peptides longer than 9 aa. In this case, the coefficients for P1-P4 might be the same as with nonamers, but the coefficients for P6-P9 would apply to P(Ω-4) - PΩ, where Ω stands for the last amino acid in the peptide. We have found that the binding properties of some peptides can be explained adequately in this way (data not shown), but many peptides are predicted very poorly, especially when there is a Gly residue at P2 or P3. So far, the longest peptide that we have tested that appears to bind by the looping-out mechanism is the 15-mer GLFGGGGGVKGGFGV, which contains favorable dominant anchor residues at P2 and PΩ, and also favorable auxiliary anchor residues at P3 and P(Ω-2). Before this extension of the IBS hypothesis will be generally useful, it will be necessary to work out an additional set of rules that takes into account the variety of peptide backbone conformations that can be used to accommodate the looped-out residues.

We have used the coefficients listed in Table V to ask whether the well-studied antigenic and endogenous peptides represent the highest affinity peptides that could be generated from their parent proteins. The calculations listed in Table VII indicate that, so far as we can tell, these biologically important peptides are usually among the top 2% of all possible HLA-A2 binding peptides. For example, the optimal HLA-A2-restricted peptide GILGFVFTL is predicted to bind more tightly to HLA-A2 than any other peptide that can be derived from the influenza matrix protein, even though it contains a relatively unfavorable Ile at P2. Thus, there is no reason to believe that antigenic peptides are preferentially selected from a lower affinity set of peptides, as has been proposed (33). It would be interesting to determine whether any of the other peptides that are predicted to form stable complexes with HLA-A2 are ever antigenic or associated with HLA-A2 in vivo. In this way, one could address the relative importance to antigenicity of peptide binding to HLA-A2 compared to other factors like protein proteolysis, protein turnover, peptide stability, peptide transport, the rate of formation of the complex, and holes in the T cell repertoire. Unlike the dissociation of the HLA-A2 complex, which is a unimolecular process, the processes that affect the rate of formation of the HLA-A2 complex are potentially subject to control mechanisms that may differ between cell types, making it much more difficult to study them. In any case, we believe that the coefficients in Table V provide the best means available so far to identify HLA-A2 binding peptides, whether or not they turn out to be antigenic, immunologically silent, or never formed in vivo. It would be interesting to determine whether a table of coefficients calculated by similar means would be able to improve the predictive power of the motifs that have been elucidated for class II binding peptides (34). Other macromolecular interactions such as ligand/antibody and oligonucleotide/DNA binding protein might also be addressed using a mathematical approach similar to that described here.

Note added in proof. Software is being developed to make the coefficients in Table V publicly accessible through the National Center for Biotechnology Information at the National Library of Medicine.

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HIV-1 gag-SPECIFIC CYTOTOXIC T LYMPHOCYTES RECOGNIZE MULTIPLE HIGHLY CONSERVED EPITOPES

Fine Specificity of the gag-Specific Response Defined by Using Unstimulated Peripheral Blood Mononuclear Cells and Cloned Effector Cells[1,2]

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CTL directed at the highly conserved HIV-1 gag protein have been described in HIV-1 seropositive persons and may be an important host defense against this retrovirus. Presently only limited data are available regarding the specific epitopes recognized by these CTL. In this study, we have performed a detailed examination of the gag-specific CTL response in three HIV-1 seropositive subjects, using both unstimulated PBMC and cloned CTL. Lysis of gag-expressing targets was found to be mediated by $CD3^+CD8^+$ lymphocytes and restricted by class I Ag. Multiple class I Ag were found to restrict gag epitopes in each subject studied, with as many as three of these Ag involved in presenting gag CTL epitopes in a single subject. The majority of gag-specific CTL activity was found to be directed against epitopes in the p24 subunit of the gag protein, with at least seven different HLA class I-restricted CTL p24 epitopes identified in these three subjects. Less CTL activity was directed against p17 subunit of gag and two CTL epitopes were identified in this protein. Although as many as four different epitopes in gag were recognized using CTL from a single subject, none of the epitopes was recognized by CTL from more than one subject. Analysis of gag epitope recognition using cloned CTL demonstrated heterogeneity and specificity not appreciated using unstimulated PBMC. The identification of multiple relatively conserved epitopes in the HIV-1 gag protein and the heterogeneity of CTL responses to this protein may have important implications for vaccine development and our understanding of AIDS pathogenesis.

Infection with HIV-1 typically results in a prolonged asymptomatic phase characterized by progressive immunodeficiency ultimately leading to opportunistic infections and death. The immunologic and virologic factors which contribute to the maintenance of this prolonged asymptomatic phase are not well defined, but it is likely that the host immune response plays an important role in inhibiting virus replication. Both humoral and cellular immune responses to HIV-1 have been described in infected persons (1–9), but the relative contributions of these arms of the immune system to protective immunity have not yet been determined. A vigorous cytotoxic T cell response to HIV-1 has been detected in infected persons (10–15), and several lines of evidence suggest that HIV-1-specific CTL are effective in inhibiting virus replication. $CD8^+$ lymphocytes with the phenotype of CTL have been demonstrated to inhibit replication of HIV-1 in vitro (16), and a decline in HIV-1-specific CTL activity has been observed in individuals who have developed AIDS (17). These observations and previous experiments demonstrating that CTL mediate protective immunity against other viral infections (18, 19) have led to the suggestion that an effective AIDS vaccine should be immunogenic for CTL epitopes (20). Further characterization of the viral epitopes recognized by HIV-1-specific CTL should therefore contribute to the rational design of an AIDS vaccine and may help define the role of CTL in the pathogenesis of HIV-1 infection.

Multiple HIV-1 proteins have been shown to be recognized by CTL including gag (10, 12, 14, 15), RT[4] (11), envelope (10, 13, 14), nef (15, 21), and vif (15). In other viral infections, CTL responses to relatively conserved structural proteins have been shown to provide cross-reactive immunity against genetically divergent viral pathogens (22, 23), and CTL to such proteins may therefore be a particularly important component of the host immune response. The HIV-1 gag protein is highly conserved among sequenced isolates, and CTL responses against the p55 gag protein have been described in a majority of HIV-1 seropositive subjects (12, 14, 15). However, only a limited number of gag CTL epitopes and their restricting Ag have been defined (12, 24, 25).

In this study we have performed a detailed analysis of

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[4] Abbreviations used in this paper: RT, reverse transcriptase; B-LCL, B-lymphoblastoid cell line; v/lac, recombinant vaccinia virus expressing β-galactosidase; v/p55, recombinant vaccinia virus expressing p55 gag; v/p24, recombinant vaccinia virus expressing the p24 gag subunit; aa, amino acid.

the gag-specific CTL responses in three HIV-1 seropositive individuals with significant activity against p55-expressing targets. By using unstimulated PBMC and CTL clones, we have examined cytolytic activity against gag subunit vectors, defined the HLA Ags restricting these responses, and identified specific CTL epitopes within the gag protein. Our results demonstrate that the gag-specific response is heterogeneous both with respect to HLA restriction and epitope specificity and is directed at multiple relatively conserved CTL epitopes.

MATERIALS AND METHODS

Patient population. Three HIV-1 seropositive subjects with significant gag-specific CTL activity were selected for study. All subjects gave written informed consent and the study was approved by the Massachusetts General Hospital Human Studies Committee. These three subjects were chosen from a group of five HIV-1 seropositive subjects who had been screened for lysis of autologous gag-expressing targets and were selected for further analysis on the basis of their exhibiting significant gag-specific activity (i.e., greater than 20% specific lysis at an E:T ratio of 100:1). gag-specific lysis of autologous targets at an E:T ratio of 100:1 was less than 10% in the two subjects who were not further studied. At the time of entry, all subjects were asymptomatic; $CD4^+$ lymphocytes were 145/mm^3 in subject 010-035l, 1150/mm^3 in subject 010-063j, and 900/mm^3 in subject 010-115l. Subject 010-035l was referred to as subject 63 in a previous report from this laboratory (26). During the course of this study (approximately 15 mo), subject 010-063j had a progressive decline in $CD4^+$ cells to less than 200/mm^3 and subsequently developed *Pneumocystis carinii* pneumonia. The other two subjects remained stable without significant declines in $CD4^+$ T lymphocytes. Serum HIV-1 p24 Ag levels were undetectable in all subjects during the study period.

Cell lines. EBV-transformed B-LCL were established and maintained as described previously (10) in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 μg/ml), and HEPES (10 mM) containing 20% (vol/vol) heat-inactivated FCS (Sigma). Allogeneic B-LCL were also obtained from the American Society for Histocompatibility and Immunogenetics B cell line repository. All cell lines were free of mycoplasma infection by standard culture techniques.

HLA typing. HLA typing was performed by the Massachusetts General Hospital Tissue Typing Laboratory using standard serological techniques.

Recombinant vaccinia viruses. Recombinant vaccinia viruses expressing the full-length p55 (v/p55), and subunit p17 (v/p17) and p24 (v/p24) gag proteins, were constructed from the BH10 plasmid of HIV-1 as previously described (6).[5] Recombinant vaccinia viruses expressing the HIV-1 RT (VCF21), envelope (PE16) and the control *lacZ* (v/lac) genes were provided by Dr. Bernard Moss.

Synthetic HIV-1 gag-peptides. Synthetic peptides corresponding to the p55 BH10 sequence were synthesized by Multiple Peptide Systems (San Diego, CA) using t-butoxycarbonyl N-protected aas and 4-methylbenzhydramine resin in the "tea bag" methodology of Houghten (27). Peptides were cleaved from the resin with anhydrous hydrogen fluoride, washed with ether, extracted with 10% acetic acid, and evaluated for purity by reverse-phase analytical HPLC. Peptides for the p17 sequence consisted of a series of peptides 25 aa in length and overlapping by 8 aa. Peptides for the p24 sequence consisted of peptides 22 aa in length and overlapping by 12 aa. All peptides were synthesized as C-terminal amides. Amino acids are numbered as indicated for the HIV-1 BH10 clone (28). Additional smaller peptides (8–17 aa) were synthesized for fine mapping using a technique similar to that described above (29). Lyophilized peptides were reconstituted at 2 mg/ml in sterile distilled water with 10% DMSO (Sigma) with or without 1 mM dithiothreitol (Sigma).

Fractionation of lymphocytes using magnetic affinity cell sorting. PBMC were separated into $CD8^+$ lymphocyte-enriched and $CD8^+$ lymphocyte-depleted fractions using an anti-CD8 mAb covalently conjugated to magnetic particles (BioMag Particles, Collaborative Research Incorporated, Bedford, MA). Following separation on Ficoll-Hypaque density gradients (Sigma), PBMC were washed twice in RPMI and then resuspended at 10^7 cells/ml in RPMI with 1% FCS. PBMC were then incubated at 4°C for 60 min with magnetic anti-CD8 antibody at a final cell concentration of 5×10^6 cells/ml and particle to cell ratio of 25:1. $CD8^+$ lymphocytes bound to magnetic particles were then separated from the remaining PBMC using a high field strength magnetic separator (Collaborative Research Incorporated). Magnetic separation was then repeated for both $CD8^+$ lymphocyte-enriched and $CD8^+$ lymphocyte-depleted cell populations, and these fractions were then suspended in RPMI with 10% heat-inactivated FCS. $CD8^+$ lymphocytes bound to magnetic particles were cultured overnight at 10^6 cells/ml and then magnetic particles removed from $CD8^+$ lymphocytes by using the magnetic separator. Cell viability for both $CD8^+$ lymphocyte-enriched and $CD8^+$ lymphocyte-depleted fractions exceeded 95%.

Generation of HIV-1-specific CTL clones. CTL clones were isolated and maintained using a modification of a technique described previously (26). Briefly, PBMC from seropositive subjects were seeded at 50, 25, or 10 cells per well in 96-well plates in a final volume of 200 μl per well of feeder cell solution containing 10^6/ml irradiated allogeneic PBMC from HIV-1 seronegative subjects in RPMI with 10% heat-inactivated FCS supplemented with 100 U/ml of human rIL-2 (Hoffman-La Roche, Nutley, NJ; I. E. Du Pont, N. Billerica, MA, or Boehringer Mannheim, Indianapolis, IN). The CD3-specific monoclonal antibody 12F6 (30) was added at 0.1 μg/ml as a stimulus to T cell proliferation. Plates were incubated at 37°C in a humidified chamber with 5% CO_2 and fed one or two times per week with partial medium exchanges. After 2 to 3 wk, the percentage of wells exhibiting growth was approximately 40 to 60% of wells plated at 50 cells/well, 15 to 35% of wells plated at 25 cells/well and 12 to 18% at 10 cells/well. Cells from wells demonstrating growth were then transferred to 24-well plates and restimulated by adding 1 ml of rIL-2-containing medium with 10^6 irradiated feeder cells/ml and anti-CD3 mAb at 0.1 μg/ml. Approximately 2 wk later clones were screened for CTL activity. Clones exhibiting CTL activity were then restimulated every 10 to 14 days by resuspending in feeder cell solution with anti-CD3 mAb. CTL clones were also generated by stimulating $CD8^+$ lymphocytes obtained by magnetic affinity cell sorting with PHA-P (5 μg/ml, Difco, Detroit, MI) in RPMI supplemented with 10% FCS and 100 U rIL-2 for 3 days, and then plating at limiting dilution in the presence of irradiated feeder cells. Clones were restimulated every 2 wk by resuspending with irradiated feeder cells supplemented with 10% PHA-P-conditioned supernatant from HIV-1 seronegative PBMC. Clones obtained in this fashion were screened as described above.

Flow cytometry analysis. Cells to be analyzed (0.5 to 1.0×10^6 cells) were washed once in PBS and then incubated with a fluorescent probe-conjugated anti-CD4 mAb, anti-CD8 mAb, or a similarly labeled control mAb (Coulter Electronics, Hialeah, FL). Following a 30-min incubation at 4°C cells were washed in PBS, fixed with 2% paraformaldehyde in PBS, mixed with an equal volume of PBS with 2% FCS and analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Cytotoxicity Assay. Target cells consisted of B-LCL infected with recombinant vaccinia or preincubated with synthetic HIV-1 gag peptides. Vaccinia-infected targets were prepared by incubating 2.5 to 10×10^6 B-LCL in log-phase growth with recombinant vaccinia at 1 to 5 plaque-forming U/cell for 16 h at 37°C. Cells were then labeled with 100 to 150 μCi of $Na_2(^{51}CrO_4)$ (New England Nuclear) for 45 to 60 min and washed three times with RPMI with 10% FCS. Peptide-coated targets were obtained by incubating 2 to 3×10^6 B-LCL with peptide (40 to 200 μg/ml) for 1 to 16 h and then labeling with 100 to 150 μCi of $Na_2(^{51}CrO_4)$ for 60 min. Cytolytic activity was determined in a standard ^{51}Cr-release assay (10) using U-bottom microtiter plates containing 10^4 targets per well. PBMC were obtained by Ficoll/Hypaque centrifugation, resuspended in RPMI with 10% FCS, and tested at E:T ratios ranging from 100:1 to 12.5:1 in a 5- to 6-h assay. CTL clones were tested at E:T ratios ranging from 10:1 to 1:1 in a 4-h assay. All assays were performed in either duplicate or triplicate. Supernatants were then harvested and counted on a Cobra Gamma Counter (Packard Instrument Company, Meriden, CT) and percent cytotoxicity determined from the formula: 100 × [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. Maximum release was determined by lysis of targets in water or detergent (1% Triton X-100, Sigma). Spontaneous release was less than 30% of maximal release unless otherwise noted.

RESULTS

CTL activity of unstimulated PBMC against HIV-1 proteins and gag-subunit vectors. Unstimulated PBMC obtained from the three HIV-1 seropositive individuals selected for this study exhibited vigorous specific cytoly-

[5] Koup, R. A., C. A. Pikora, K. R. Luzuriga, G. P. Mazzara, D. L. Panicali, and J. L. Sullivan. Limiting dilution analysis of cytotoxic T lymphocytes to human immunodeficiency virus gag antigens in infected persons: quantitation of effector cell populations with p17 and p24 specificities. *Submitted for publication.*

TABLE II

gag-specific CTL activity is mediated by CD8⁺ lymphocytes

Subject	Percent Specific Lysis[a]					
	Unfractionated PBMC[b]		CD8⁺ lymphocyte-enriched PBMC[c]		CD8⁺ lymphocyte-depleted PBMC[d]	
	Lac	Gag	Lac	Gag	Lac	Gag
010-035l	0	61	0	62	6	15
010-115l	7	28	4	24	7	6

[a] A total of 10^4 targets were labeled with ^{51}Cr and incubated with effector cells for 5 to 6 h. The E:T ratio for all assays was 50:1 except for CD8⁺ lymphocyte-enriched PBMC from subject 010-115l which were tested at 40:1. Targets consisted of autologous B-LCL expressing either p55 gag or lac control. Spontaneous release varied from 12 to 26%.

[b] Unfractionated PBMC were prepared by Ficoll-Hypaque centrifugation.

[c] CD8⁺ lymphocyte-enriched PBMC were isolated from unstimulated PBMC using magnetic anti-CD8 mAb and contained 1 to 2% CD4⁺ lymphocytes.

[d] CD8⁺-lymphocyte depleted PBMC were prepared from unstimulated PBMC by removing CD8⁺ lymphocytes with magnetic anti-CD8 mAb. Residual CD8⁺ lymphocytes were <1% for subject 010-115l and 9% for subject 010-035l.

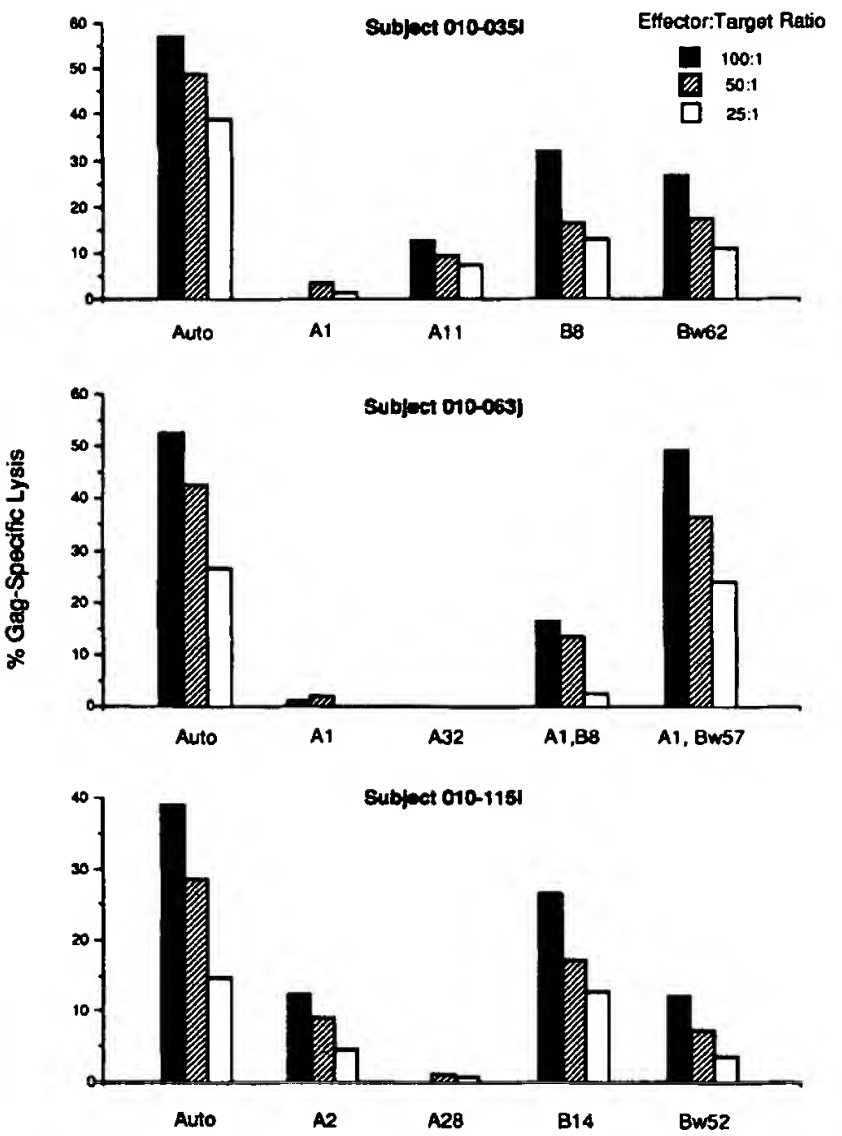

Figure 1. gag-specific cytoloysis by unstimulated PBMC is restricted by HLA class I Ag. Autologous (*Auto*) or allogeneic targets matched at the indicated HLA class I Ag were infected with recombinant vaccinia expressing either the HIV-1 p55 gag gene or control *lacZ* protein. gag-specific lysis was calculated for each target tested by subtracting the specific lysis of lac-expressing targets from the specific lysis of gag-expressing targets. gag-specific cytotoxicity was simultaneously tested for all targets for each subject in a 6-h ^{51}Cr release assay by using fresh unstimulated PBMC as effector cells at E:T ratios of 100:1, 50:1, and 25:1. Spontaneous release varied from 12 to 27%. Cytolytic activity against v/lac infected targets ranged from 2 to 6% for autologous targets and 0 to 24% for allogeneic targets. Complete HLA types for the three subjects are as follows: 010-035l, A1, A11; B8, Bw62; Cw4; DR3, Drw52, DQw2; 010-063J, A1, A32; B8, Bw57; Bw4, Bw6; Cw6, Cw7; DR2, DR3, Drw52, DQw1, DQw2; 010-115l, A2, A28; B14, Bw52; Bw4, Bw6; DR1, DR2, DQw1.

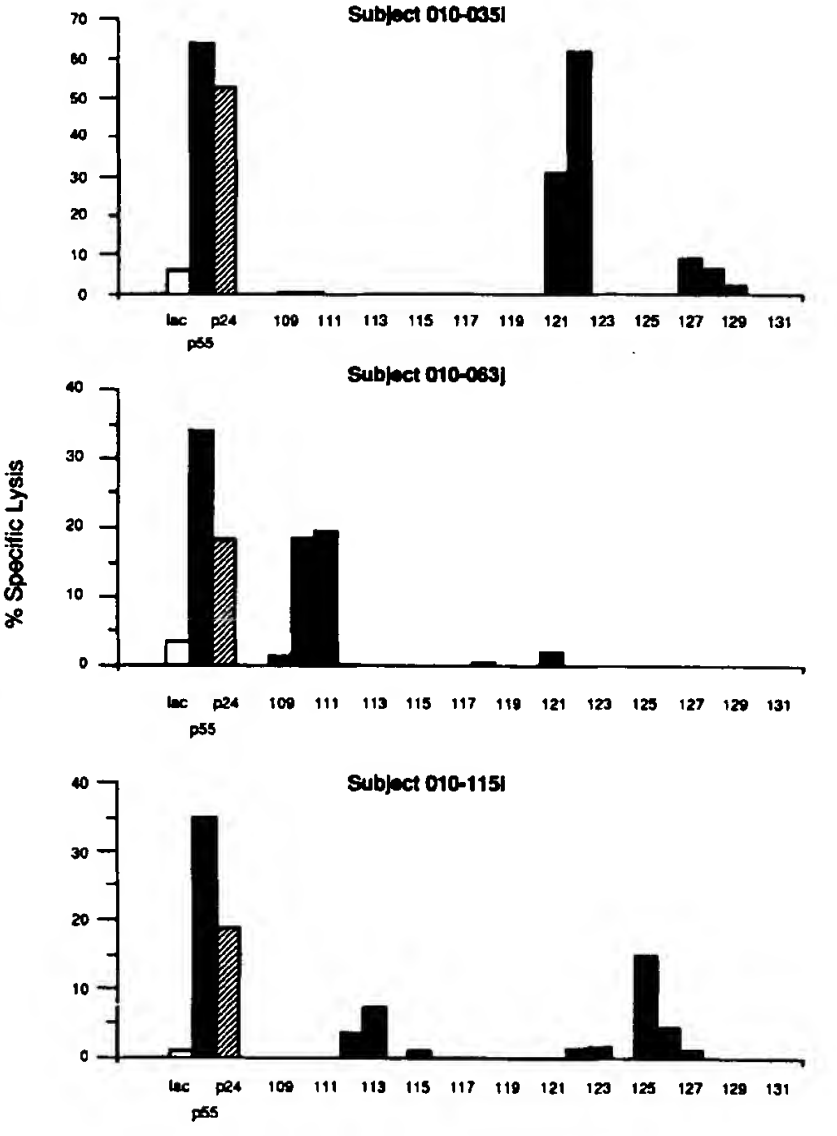

Figure 2. Epitope specificity of gag-specific CTL in unstimulated PBMC. Autologus B-LCL were either infected with recombinant vaccinia expressing the control *lacZ* (*lac*), HIV-1 p55 (*p55*), p24 (*p24*), or incubated with a panel of overlapping synthetic peptides corresponding to the HIV-1 p24 sequence. After labeling, B-LCL were then used as targets in a 6-h ^{51}Cr-release assay by using fresh unstimulated PBMC at an E:T ratio of 100:1. Spontaneous release varied from 12 to 27%. All targets were tested simultaneously for each subject. Amino acid sequences for the peptides containing the dominant epitopes for each individual are: p24/110 (aa 143–164) VHQAISPRTLNAWVKVVEEKAF; p24/111 (aa 153–174) NAWVKVVEEKAFSPEVIPMFSA; p24/121 (aa 253–274) NPPIPVGEIYKRWIILGLNKIV; p24/122 (aa 263–284) KRWIILGLNKIVRMYSPTSILD; p24/125 (aa 293–314) FRDYVDRFYKTLRAEQASQEVK; p24/126 (aa 303–324) TLRAEQASQEVKNWMTETLLVQ.

been observed using bulk unstimulated PBMC, the majority of these clones recognized p24. In addition, two clones (35E3 and 115Q6) were isolated which lysed p17-expr ssing targets. Phenotypic analysis of gag-specific CTL clones revealed them to be $CD3^+CD4^-CD8^+$ (data not shown).

HLA restriction of gag-specific CTL clones. To define the HLA restriction of gag-specific CTL clones, cytolytic activity was tested against a panel of allogeneic B-LCL infected with v/p55 and matched at various class I HLA Ag. Consistent with their $CD3^+CD8^+$ phenotype, all CTL

TABLE III

Cytolytic activity of gag-specific CTL clones

Subject	Clone	Percent Specific Lysis[a]						
		lac[b]	p55	p24	p17	RT	Env	K562
010-035I	35E3	1	35	6	31	9	2	ND
	35G26	3	39	52	5	0	4	ND
	35J18	1	39	31	2	ND	3	ND
010-063J	63C21	0	73	74	2	1	0	9
	63F15	0	45	46	2	0	0	0
010-115I	115A19	0	60	80	1	0	0	0
	115G18	0	73	79	0	3	0	ND
	115M15	0	57	67	0	2	0	ND
	115N2	0	42	62	0	1	0	3
	115Q6	3	52	8	37	2	1	ND

[a] A total of 10^4 target cells were labeled with ^{51}Cr and incubated with effector cells for 4 h. The E:T ratio for all assays was 10:1 except clone 35J18 which was tested at 2:1. Spontaneous release varied from 7 to 26%.

[b] Target cells were prepared from autologous B-LCL infected for 16 to 18 h with recombinant vaccinia expressing the control *lacZ* gene (lac), HIV-1 p55 (p55), p24 (p24), p17 (p17), RT, or envelope (Env) proteins, or the NK-sensitive cell line K562.

TABLE IV

gag-specific CTL clones from subject 010-115I are HLA class I restricted

Shared HLA Ag of Target Cell[a]	Percent gag-Specific Lysis[b]				
	115A19	115G18	115M15	115N2	115O6
Auto	62	73	53	43	57
A2	2	0	0	1	42
A28	0	0	0	0	ND
B14	0	34	36	25	2
Bw52	23	0	0	0	0

[a] Targets consisted of autologous (Auto) or allogeneic B-LCL matched at indicated HLA Ag which were infected with either v/p55 gag or v/lac. The restriction pattern of clone 115A19 was confirmed with two different Bw52-matched targets.

[b] A total of 10^4 targets were labelled with ^{51}Cr and incubated with effector cells at an E:T ratio of 10:1 for 4 h except for clone 115Q6 for which the E:T ratio was 5:1. gag-specific lysis was calculated for each target by subtracting the specific lysis of lac-expressing targets from the specific lysis of gag-expressing targets. Spontaneous release varied from 6 to 25%. Lysis of lac-expressing target cells ranged from 0 to 5%.

clones were found to be class I restricted (Tables IV and V). In each case, the HLA Ag restricting these clones were Ag which had been shown to restrict lysis when bulk PBMC from the same donor were used as effector cells. For example, the majority of CTL clones from subject 010-115I were restricted by HLA B14, the dominant restricting Ag for unstimulated PBMC from this subject, and each of the remaining clones isolated from this subject (115A19 and 115Q6) was restricted by one of the minor restricting Ag, Bw52 and A2, respectively. The HLA restriction pattern of CTL clones from the other two subjects was also similar to that observed using their unstimulated PBMC.

Epitope mapping of gag-specific CTL clones. Mapping of specific epitopes recognized by gag-specific CTL clones was performed using autologous targets cells incubated with one of a series of overlapping peptides corresponding to either the HIV-1 p17 or p24 sequence. The majority of p24-specific clones recognized peptides which were also recognized by the unstimulated PBMC from the same subject. Thus, of the two p24-specific CTL clones obtained from subject 010-035I, one clone was specific for each of the two dominant peptides which sensitized targets for lysis by PBMC from this subject (Table VI). Similarly, CTL clones obtained from subject 010-063J recognized one of the two peptides which were recognized by autologous PBMC (Table VII). However, each clone was specific for only one of the two adjacent overlapping peptides, even though both shared the same HLA restricting element. All of the HLA B14-restricted, p24-specific clones obtained from subject 010-115I recognized p24/125, the dominant peptide recognized by fresh unstimulated PBMC from this subject, but recognition of the adjacent peptide p24/126 varied among clones, ranging from approximately equal levels of lysis by clone 115 M15 to no significant recognition by 115G18 (Table VIII).

Epitope specificity for each of the p17-specific CTL clones was defined by examining lysis of autologous targets incubated with a panel of overlapping peptides corresponding to the entire p17 sequence (Table IX). Each clone recognized a different epitope: the Bw62-restricted clone 35E3 lysed only targets incubated with peptide p17/2 (aa 18–42), whereas the A2-restricted clone 115Q6

TABLE V

gag-specific CTL clones from subjects 010-035I and 010-063J are HLA class I restricted

Percent gag-specific lysis[a] Subject 010-035I			*Percent gag-specific lysis* Subject 010-063J		
Shared HLA Ag of target cell[b]	35E3	35G26	Shared HLA Ag of target cell	63JC21	63JF15
Auto	32	36	Auto	33	25
A1	0	0	A1, DR2	0	2
B8	0	64	A1, B8, DR3	1	0
A11	0	0	A32	0	0
Bw62	22	0	A1, Bw57	26	17

[a] A total of 10^4 targets were labeled with ^{51}Cr and incubated with effector cells at an E:T ratio of 10:1 for 4 h. gag-specific lysis was calculated for each target tested by subtracting the specific lysis of lac-expressing targets from the specific lysis of gag-expressing targets. Spontaneous release varied from 7 to 32%. Lysis of lac-expressing target cells ranged from 0 to 8%.

[b] Targets consisted of autologous (Auto) or allogeneic B-LCL matched at indicated HLA Ag which were infected with either v/p55 or v/lac.

TABLE VI

Epitope mapping of p24-specific CTL clones from subject 010-035i

Peptide	aa	Percent Specific Lysis[a]	
		35G26	35J18
p24/120	243–264	3	1
p24/121	253–274	87	2
p24/122	263–284	4	36
p24/123	273–294	3	0
p24/127	313–334	3	2
p24/128	323–344	1	ND

[a] A total of 10^4 targets were labeled with ^{51}Cr and incubated with effector cells for 4 h. The E:T ratio for 35G26 was 10:1 and 35J18 was 2.5:1. Targets consisted of autologous B-LCL incubated with the indicated peptide for 1 h at a final concentration of 100 μg/ml. Spontaneous release varied from 12 to 22%.

TABLE VII

Epitope mapping of p24-specific CTL clones from subject 010-063j

Peptide	aa	Percent Specific Lysis[a]	
		63JC21	63JF15
p24/109	133–154	1	0
p24/110	143–164	0	16
p24/111	153–174	16	0
p24/112	163–184	0	1

[a] A total of 10^4 targets were labeled with ^{51}Cr and incubated with effector cells for 4 h. The E:T ratio for all clones was 10:1. Targets consisted of autologous B-LCL incubated with the indicated peptide for 1 h at final concentration of 100 μg/ml. Spontaneous release varied from 8 to 15%.

TABLE VIII

Epitope mapping of p24-specific HLA B14-restricted CTL clones from subject 010-115i

Peptide	aa	Percent Specific Lysis[a]		
		115G18	115M15	115N2
p24/124	283–304	0	0	0
p24/125	293–314	40	23	84
p24/126	303–324	2	17	29
p24/127	313–334	0	0	1

[a] A total of 10^4 targets were labeled with ^{51}Cr and incubated with effector cells for 4 h. Clones were tested at an E:T ratio of 10:1 against all targets simultaneously. Targets consisted of autologous B-LCL incubated with the indicated peptide for 16 h at a final concentration of 40 μg/ml. Spontaneous release varied from 20 to 24%.

TABLE IX

Epitope mapping of p17-specific CTL clones

Peptide	aa	Percent Specific Lysis[a]	
		35E3	11596
p17/1	1–25	0	3
p17/2	18–42	44	6
p17/3	35–59	0	4
p17/4	52–76	0	3
p17/5	69–93	1	64
p17/6	86–110	1	4
p17/7	103–127	0	1
p17/8	120–144	0	1

[a] A total of 10^4 targets were labeled with ^{51}Cr and incubated with effector cells at an E:T of 5:1 for 4 h. Targets consisted of autologous B-LCL incubated with the indicated peptide for 1 h at a final concentration of 100 μg/ml. The sequence of peptide p17/2 is KIRLRPGGKKKYKLK-HIVWASRELE and of p17/5 is QTGSEELRSLYNTVATLYCVHQRIE. Spontaneous release varied from 13 to 24%.

lysed targets sensitized with peptide p17/5 (aa 69–93).

Peptide sensitized targets did not readily identify the epitope recognized by a CTL clone in all instances. Despite significant lysis of autologous targets infected with v/p55 by the p24-specific clone 115A19 (see Table III), initial screening with this clone against a panel of target cells incubated with p24 peptides yielded only low level (<10%) but reproducible lysis against a single peptide (data not shown). After overnight incubation of autologous targets with individual peptides at 200 μg/ml, significant lysis of targets sensitized with peptide p24/115 was observed, although still significantly less than that demonstrated against targets infected with v/p55 (Figure 3). The reason that a prolonged incubation period with a high concentration of peptide was required to sensitize target cells for lysis by this particular clone remains unknown.

HLA restriction of p24 peptides recognized by unstimulated PBMC. By identifying the restricting Ag of CTL clones which were specific for peptides recognized by PBMC from each subject, we were able to demonstrate the restricting Ag for the majority of dominant p24 peptides recognized by unstimulated PBMC. In two cases, additional experiments using unstimulated PBMC as effector cells were conducted to define the HLA restriction of specific peptides. The majority of gag-specific CTL activity in PBMC from subject 010-035i was directed against the two adjacent peptides p24/121 and p24/122, a finding compatible with either a single epitope in the overlap region between the two peptides or two epitopes in nonoverlapping regions of the adjacent peptides. However, analysis of the epitope specificity of CTL clones obtained from this subject demonstrated that there are two separate epitopes contained in these peptides, each clone recognizing only one of these overlapping peptides (see Table VI). The HLA Ag restriction of clone 35J18, which recognizes the peptide p24/122, could not be definitively shown due to the inability to propagate this clone in long term culture. Experiments using PBMC from subject 010-035i demonstrated that recognition of peptide p24/122 was Bw62 restricted and confirmed that the two peptides contained distinct epitopes (Fig. 4). Lysis of HLA-matched, allogeneic peptide-sensitized targets by unstimulated PBMC was also used to define the HLA restriction of peptide p24/113, one of the peptides which was recognized by PBMC from subject 010-115i. CTL assays using unstimulated $CD8^+$ cells from subject 010-115i obtained by magnetic affinity cell sorting showed that recognition of peptide p24/113 was restricted by HLA B14 (data not shown), the same Ag which restricted lysis of peptide p24/125 in this subject. Although recognition of peptide p24/127 by PBMC from subject 010-035i was confirmed in subsequent assays, no CTL clones specific for this peptide were obtained, and the HLA restriction of this peptide was not defined.

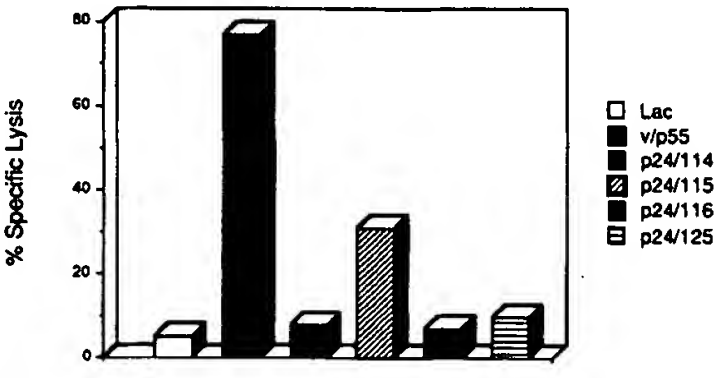

Figure 3. Identification of the epitope for the Bw52-restricted CTL clone 115A19. Autologous B-LCL were either infected with recombinant vaccinia expressing the control *lacZ* protein (lac) or HIV-1 p55 (p55), or incubated with synthetic peptides for 16 hours at 200 μg/ml. Peptide p24/114 corresponds to aa 183 to 204, p24/115 aa 193 to 214, p24/116 aa 203 to 224 and p24/125 to aa 293 to 314. The aa sequence of peptide p24/115 is GHQAAMQMLKETINEEAAEWDR. After labeling, B-LCL were then used as targets in a 4-hour release assay using the CTL clone 115A19 at an E/T ratio of 5:1. Spontaneous release varied from 7 to 14%.

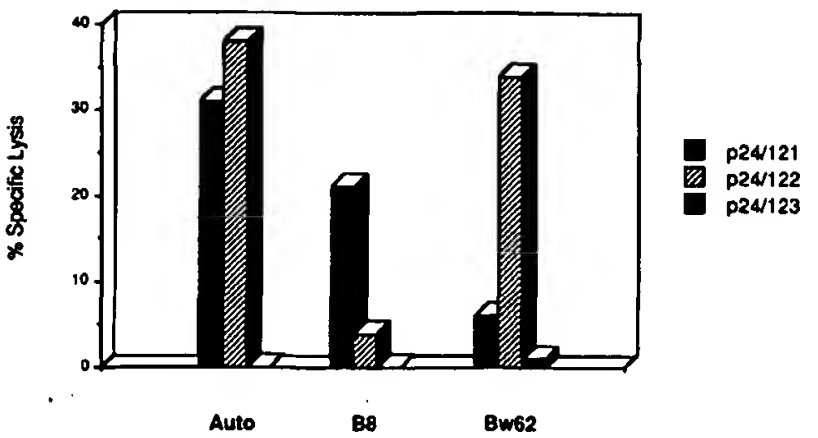

Figure 4. The overlapping peptides p24/121 and p24/122 contain two distinct CTL epitopes which are restricted by different HLA Ag. Target cells were prepared from autolgous (*Auto*) or allogeneic B-LCL matched at HLA class I Ag and incubated with the indicated peptide at 100 μg/ml for 1 h. Effector cells consisted of fresh unstimulated PBMC obtained from subject 010-035l. A total of 10^4 target cells were labeled with ^{51}Cr and incubated with effector cells at an E:T ratio of 100:1 for 5 h. Spontaneous release varied from 15 to 25%.

Fine mapping of the B14-restricted p24 gag epitope. Two CTL clones specific for the B14-restricted peptide p24/125 could be maintained in long term culture, thus permitting fine mapping of this epitope. CTL clones 115 M15 and 115N2 were tested against a panel of autologous targets cells sensitized with synthetic peptides (Table X). Peptides were synthesized with sequential truncations at either the N-terminal or C-terminal end of the putative epitope defined by the overlap between peptides p24/125 and p24/126. The minimal epitope suggested by these results is the 9-aa sequence RAEQASQEV corresponding t aa 305–313. As anticipated from the results obtained using the panel of overlapping 22 aa peptides (Table VIII), this epitope is entirely contained within the overlap region shared by peptides p24/125 and p24/126.

DISCUSSION

The present study provides a detailed analysis of the fine specificity of gag-specific CTL responses in three HIV-1 seropositive individuals. As demonstrated previously (12, 14, 15), the gag-specific CTL response was found to be mediated by $CD3^+CD8^+$ lymphocytes which are HLA class I restricted. The majority of the gag-specific CTL response was directed against p24, although a minor response was directed against p17. Similar results have been obtained using precursor frequency analysis of unstimulated PBMC obtained from HIV-1 seropositive hemophiliacs, although an increased percentage of p17-specific CTL was found in memory CTL (see footnote 5). Lower levels of gag-specific CTL activity reported previously from this laboratory (10, 26) are related to the recombinant vector used in the earlier studies (R. P. Johnson, D. Kuritzkes, B. D. Walker, unpublished observations).

The epitope specificity and HLA restriction of CTL clones paralleled in large part the activity seen with unstimulated PBMC. All clones isolated were restricted by HLA Ag which restricted gag-specific cytolysis by donor PBMC, and CTL clones were obtained from each subject which recognized the dominant peptides recognized by bulk PBMC. In several instances, however, analysis of epitope specificity of CTL clones revealed fine distinctions not readily apparent from data obtained using PBMC. CTL clones obtained by the technique used in this study, which does not involve in vitro stimulation with viral Ag, thus constitute a valuable tool to analyze the CTL response in HIV-1 infected individuals, in that these clones reflect Ag specificity determined in vivo and can be used to analyze cellular immune responses in detail.

A remarkable feature of the gag-specific CTL activity observed in these subjects is the heterogeneity of responses, both with respect to HLA restriction patterns and recognition of specific epitopes. With regard to HLA restriction, multiple HLA types were observed to present gag epitopes. In each of these three subjects, two or three class I Ag were found to restrict gag-specific CTL activity in PBMC, and a total of seven restricting Ag were identified. A similar diversity of HLA restriction was observed previously with HIV-1 RT-specific CTL clones (26).

The heterogeneity of CTL responses was also reflected in the epitope specificity of gag-specific CTL. A total of nine HIV-1 gag synthetic peptides were found to sensitize targets for recognition by class I-restricted CTL from these three seropositive subjects (Table XI). (Peptide p24/127 was recognized by unstimulated PBMC from subject 010-035, but the restricting HLA Ag was not determined.) Two of the three subjects recognized at least four epitopes in the gag protein; for each of these subjects, three epitopes in p24 and one in p17 were identified. In the remaining subject we were unable to determine if the CTL were directed against two nonoverlapping epitopes

TABLE X

Fine mapping of the HLA B14-restricted p24 epitope using CTL clones from subject 010-115l

Peptide	Sequence	Percent Specific Lysis[a]			
		115M15		115N2	
		3:1	1:1	3:1	1:1
125A	DRFYKTLRAEQASQEVK	55	32	43	35
125B	FYKTLRAEQASQEVK	56	35	29	26
125C	YKTLRAEQASQEVK	74	43	54	43
125D	KTLRAEQASQEVK	54	35	27	20
125E	TLRAEQASQEVK	20	16	9	7
125F	LRAEQASQEVK	56	25	47	26
125G	RAEQASQEVK	58	25	71	43
125K	AEQASQEVK	ND	ND	3	0
125L	EQASQEVK	ND	ND	0	0
125H	DRFYKTLRAEQASQEV	45	27	31	23
125l	DRFYKTLRAEQASQE	0	0	1	1
125J	DRFYKTLRAEQASQ	0	1	0	0

[a] A total of 10^4 targets were labeled with ^{51}Cr and incubated with effector cells for 4 h at E:T ratios of 3:1 and 1:1. Targets consisted of autologous B-LCL incubated with the indicated peptide for 60 min at a final concentration of 100 μg/ml. Spontaneous release varied from 10 to 15%. Peptide 125A corresponds to aa 298–314 of the HIV-1 gag protein.

TABLE XI

HIV-1 gag synthetic peptides recognized by CTL from three HIV-1 seropositive subjects

Subject	Specificity	HLA	Peptide	aa	Sequence
010-035i	p-	Bw62	p17/2	18–42	KIRLRPGGKKKYKLKHIVWASRELE
	p17	B8	p24/121	253–274	NPPIPVGEIYKRWIILGLNKIV
	p24	Bw62	p24/122	263–284	KRWIILGLNKIVRMYSPTSILD
	p24				
010-063j	p24	Bw57	p24/110	143–164	VHQAISPRTLNAWVKVVEEKAF
	p24	Bw57	p24/111	153–174	NAWVKVVEEKAFSPEVIPMFSA
010-115i	p17	A2	p17/5	69–93	QTGSEELRSLYNTVATLYCVHQRIE
	p24	B14	p24/113	173–194	SALSEGATPQDLNTMLNTVGGH
	p24	Bw52	p24/115	193–214	GHQAAMQMLKETINEEAAEWDR
	p24	B14	p24/125G	305–314	RAEQASQEVK

contained in adjacent peptides or if this discrepancy reflected heterogeneous responses to the overlap region common to the two peptides. The limited ability to propagate clones from this subject in vitro has precluded a definitive answer to this question.

Several of the epitopes described in this study are similar to CTL epitopes described previously, but with some important distinctions. The B8-restricted peptide p24/121 (aa 253–274) recognized by CTL from subject 010-035i contains a B8-restricted epitope, aa 253–267 (sequence numbering modified to correspond with (28)) recently described by Gotch et al. (25). Targets incubated with this peptide were not lysed by unstimulated PBMC obtained from the other B8-positive subject in this study, subject 010-063j, despite the presence of a vigorous gag-specific response. Another peptide which was found to sensitize targets for lysis by gag-specific CTL in this study includes a gag CTL epitope described previously, although the HLA restricting element differs. The Bw62-restricted peptide p24/122 (aa 263–284), recognized by PBMC from subject 010-035i, includes the B27-restricted p24 epitope identified by Nixon et al. (12), aa 263–277 (numbering modified to correspond with (28)). Other peptides containing CTL epitopes have been shown to bind to more than one HLA molecule (32, 33), and as increasing numbers of CTL epitopes are discovered, it appears likely that more peptides will be identified which may be presented by multiple HLA types.

We have observed both in the present study and in a prior analysis of HIV-1 RT CTL epitopes (26) that CTL from a single person may recognize multiple epitopes within a given protein and that the CTL epitopes within a given protein are likely to differ in different subjects. These observations suggest that there are likely to be a relatively large number of CTL epitopes. However, prior reports have noted that CTL responses in immunized or infected animals were directed against a relatively limited number of epitopes, and that CTL responses in an individual were likely to be directed against a single immunodominant epitope. For example, studies of HIV-1 envelope-specific CTL in mice immunized with a recombinant vaccinia virus expressing HIV-1 gp160 (34) and gag-specific CTL in SIV-infected macaques with a common MHC type (35) documented that CTL responses specific for a given protein in these animals were directed predominantly at a single epitope. An analysis of mice infected with influenza identified only two dominant CTL epitopes in the influenza hemagglutinin, and neither of these epitopes was recognized by several species of mice with unrelated MHC haplotypes (36). In contrast, our current analysis of gag-specific responses shows that one individual may recognize as many as four different CTL epitopes in the gag protein and that even unstimulated PBMC may exhibit significant lysis against multiple gag epitopes. Indeed, our comprehensive mapping of CTL epitopes in seropositive subjects has identified up to 10 HIV-1 epitopes which are recognized by CTL from a single subject (four in gag, four in RT, and two in envelope; R. P. Johnson and B. D. Walker, unpublished data). A comparable degree of heterogeneity in epitope specificity was also noted in a recent analysis of envelope-specific $CD4^+$ CTL obtained from HIV-1 seronegative subjects who were immunized with a recombinant gp160 vaccine (37).

The gag CTL epitopes identified in the present study are relatively conserved among HIV-1 isolates. For example, among sequenced HIV-1 isolates the B14-restricted epitope, aa 305–313, exhibits conservative aa substitutions in two positions (28). Although it appears likely that CTL specific for these conserved gag epitopes will recognize sequences from a variety of HIV-1 isolates, prior studies of the consequences of aa variation in HIV-1 epitopes have shown variable effects on recognition by human HIV-1-specific CTL. Hammond et al. (38) noted that even single conservative aa substitutions may alter CTL specificity for HIV-1 epitopes. However, Nixon et al. (39) showed that B27-restricted CTL which recognize the epitope 263–277 also recognize target cells incubated with the peptide corresponding to the HIV-2 sequence, which differed in 5 of the 15 aa.

Several features of p24 make it an attractive candidate for inclusion in a subunit vaccine. The identification of multiple epitopes in p24 and the presentation of these epitopes by multiple HLA types suggest that a vaccine which included p24 gag would have the potential to induce cellular immune responses in a high percentage of recipients. The potential effectiveness of p24 in eliciting cellular immune responses is further supported by the identification of several Th epitopes in this protein (40). The diversity of gag epitopes and their association with different HLA molecules is likely to limit the effectiveness of a single peptide to induce CTL responses in genetically diverse populations. However, a pool of several HIV-1 peptides, each of which can be presented by multiple HLA Ag, might be able to overcome this limitation.

Antigenic variation within critical epitopes recognized by the host immune system has been proposed as a mechanism by which HIV may escape host responses (41–43). Antigenic variation within a CTL epitope has been demonstrated to occur in mice transgenic for a virus-specific TCR who were infected with lymphocytic choriomeningitis virus (41). On the basis of studies in inbred mice strains which suggested that only a limited

number of CTL epitopes existed, other investigators have suggested that mutation in a relatively few epitopes might lead to escape fr m the cellular immune response (44, 45). In contrast, our results demonstrate that CTL from humans infected with HIV-1 are able to recognize multiple epitopes, even within a single protein. However, the ability of single aa changes to abrogate recognition by CTL of HIV-1 epitopes (38, 45) and the observation that aa variation may occur even within relatively conserved epitopes (46), suggest that antigenic variation may be an important mechanism of immune evasion by HIV-1 from the host cellular immune response. The availability of CTL clones with defined epitope specificity should facilitate studies designed to examine the role of viral sequence variation in escape from immune recognition.

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